Photoinhibition and recovery in a herbicide-resistant mutant from *Glycine max* (L.) Merr. cell cultures deficient in fatty acid unsaturation

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Abstract Photoinhibition and recovery were studied in two photosynthetic cell suspensions from soybean (*Glycine max* L. Merr): the wild type (WT) and the herbicide-resistant D1 mutant STR7. This mutant also showed an increase in saturated fatty acids from thylakoid lipids. STR7 was more sensitive to photoinhibition under culture conditions. In vivo photoinhibition experiments in the presence of chloramphenicol, in vitro studies in isolated thylakoid membranes, and immunoblot analysis indicated that the process of light-induced degradation of the D1 protein was not involved in the response of STR7 to light. At growth temperature (24°C), the recovery rate of photoinhibited photosystem II (PSII) was slower in STR7 relative to WT. Photoinhibition and recovery were differentially affected by temperature in both cell lines. The rates of photoinhibition were faster in STR7 at any temperature below 27°C. The rates of PSII recovery from STR7 were more severely affected than those of WT at temperatures lower than 24°C. The photoinhibition and recovery rates of WT at 17°C mimicked those of STR7 at 24°C. *In organelle* translation studies indicated that synthesis and elongation of D1 were substantially similar in both cell lines. However, sucrose

gradient fractionation of chloroplast membranes demonstrated that D1 and also other PSII proteins such as D2, OEE33, and LCHII had a reduced capability to incorporate into PSII to yield a mature assembled complex in STR7. This effect may become the rate-limiting step during the recovery of photoinhibited PSII and may explain the increased sensitivity to high light found in STR7. Our data may hint at a possible role of fatty acids from membrane lipids in the assembly and dynamics of PSII.

Keywords Cell culture · Fatty acid unsaturation · *Glycine* · Herbicide resistance · Light stress · Photosystem II

Abbreviations

DCBQ 2,6-Dichloro *p*-benzoquinone

DM Dodecyl-β-D-maltoside

DTT Dithiothreitol

HL High light

LHCII Light-harvesting complex II

LL Low light

OEE Oxygen-evolving extrinsic proteins

PAGE Polyacrylamide gel electrophoresis

PG Phosphatidylglycerol

PSII Photosystem II

Q_A *and* Q_B Secondary quinone electron acceptors from PSII

RC Reaction center

SDS Sodium dodecyl sulfate

WT Wild type

Introduction

Photosystem II (PSII) is a membrane-bound multiprotein complex present in all oxygenic photosynthetic organisms. This complex catalyzes the essential reactions of photosynthesis, driving electrons from water to plastoquinone and generating the proton gradient necessary for ATP production. The "heart" of this complex is the PSII reaction center (RC). The RC is composed of a heterodimer of two proteins, D1 and D2, that provide the protein backbone to which several

cofactors, such as chlorophylls, pheophytins, carotenes, Fe, Mn, and quinones, are attached (Zouni et al. 2001). One of the most outstanding features of PSII is the unusually high rate of light-dependent turnover of the D1 subunit (Matoo et al. 1981). D1 turnover is in fact a very complex phenomenon during which two different processes take place simultaneously. On the one hand, there is the photo-inactivation process caused by the light-induced damage to the D1 protein (Aro et al. 1993). On the other hand, there is the "recovery" process that involves several steps: (i) removal of the light-inactivated D1 protein from the PSII complex by proteolysis; (ii) de novo synthesis of the D1 protein precursor; (iii) sorting of the pre-D1 protein into the PSII complex; (iv) maturation of the pre-D1 protein by processing; and (v) reconstitution of the oxygen-evolving complex and recovery of a fully active PSII complex (van Wijck et al. 1995; Zhang et al. 1999). These two pathways require a fine adjustment between photo-degradation of D1 protein, de novo synthesis, and assembly into PSII to maintain a proper functioning of this photosystem. Several factors may influence this fine adjustment in vivo. Exposure of photosynthetic organisms to prolonged illumination with high light intensities increases the amount of photo-degraded D1 protein, affecting D1 protein turnover; fewer PSII units remain active and the photosynthetic activity decreases (Aro et al. 1993). The extent of photoinhibition in vivo is also temperature dependent (Tyystjärvi and Aro 1996). However, temperature affects photo-inactivation and recovery of the photosynthetic activity differentially. The latter is strongly temperature dependent (Gombos et al. 1994), while photo-damage to D1 and PSII inactivation are substantially independent of temperature (Tyystjärvi and Aro 1996). However, not only light and temperature affect D1 turnover. In cyanobacteria, oxidative and salt stress affect the repair of PSII acting on the translation of the *psbA* mRNA and the elongation of the D1 protein (Nishiyama et al. 2001; Allakhverdiev et al. 2002).

Some herbicide-resistant mutants isolated from plants and cyanobacteria presented an increased sensitivity to photoinhibition (Kirilovsky et al. 1989; Sundby et al. 1993; Constant et al. 1996; Alfonso et al. 1996). It was proposed that either conformational changes in the mutated D1 protein, rendering it more susceptible to degradation, or changes in the Q_A/Q_B electron transport rate that favored the accumulation of reduced Q_A^-, may be involved in the increased sensitivity to light (Kirilovsky et al. 1989; Sundby et al. 1993). However, these hypotheses contradicted the results obtained in other herbicide or site-directed D1 mutants (Nixon et al. 1995; Constant et al. 1996; Mulo et al. 1998).

Photoinactivation and recovery were also studied in mutants of the cyanobacterium *Synechocystis* PCC 6803 and *Arabidopsis* plants defective in the desaturation of thylakoid lipids. In cyanobacteria,

the overall thylakoid fatty acid composition has been directly related to the capacity for D1 replacement and to the rate of recovery from photoinhibition at low temperatures (Gombos et al. 1992, 1994; Kanervo et al. 1995; Sippola et al. 1998). In the cyanobacterial mutants, perturbations in D1-processing were linked to the sensitivity to light at low temperatures, leading to an accumulation of unprocessed precursor D1 protein (Kanervo et al. 1997). In higher plants, the extent of photoinhibition and recovery in vivo has also been correlated with the level of unsaturation of membrane lipids, but the mechanism remains unknown. Moon et al. (1995) found that unsaturation of the high-melting-point phosphatidylglycerol (PG) fraction in thylakoid membranes from transgenic tobacco plants stabilized the photosynthetic machinery against low-temperature photoinhibition. The possibility that reduced recovery from photoinhibition contributes to plant chilling sensitivity has been discussed (Moon et al. 1995; Somerville 1995). More recently, different *Arabidopsis* single mutants defective in *fad5* or *fad6* and a *fad3-2, fad7-2, fad8* triple mutant were found to be more sensitive to photoinhibition, the triple mutant being the most sensitive (Vijayan and Browse 2002). Recovery of PSII activity after high-light exposure was impaired in this triple mutant, but no analysis of the mechanism involved in this response was carried out.

We have previously described a new atrazine-resistant mutant, STR7, isolated from photosynthetic cell suspensions of soybean, with a single mutation in the *psbA* gene (Alfonso et al. 1996). This mutation induced the substitution of S268 to P in D1, different from the S264G or T mutation reported in other plant resistant biotypes. The mutant had reduced electron transport rates, slower growth and reduced oxygen-evolving activities than the wild type (WT), together with a higher sensitivity to light stress (Alfonso et al. 1996). In addition to this, the chloroplast membrane of STR7 accumulated an unusually high content (20%) of saturated C16:0 and reduced levels of C16:1 and C18:3 unsaturated fatty acids compared to the WT (Alfonso et al. 2001). Changes in fatty acid unsaturation have also been reported in many other atrazine-resistant mutants from plants (Pillai and St. John 1981; Lemoine et al. 1986; De Prado et al. 1992; Schwenger-Erger et al. 2002). The relationship between the *psbA* mutation and fatty acid unsaturation remains unclear, but it could be related to the influence of photosynthetic electron transport on the activity of fatty acid desaturases (Kis et al. 1998). The aim of this work was to study the photoinhibition and recovery processes in the WT and the STR7 mutant, and especially to identify the rate-limiting step that determines the high-light-sensitivity phenotype found in STR7. For this study, we followed previous work carried out in cyanobacteria and in vitro studies on isolated chloroplasts (Kanervo et al. 1995; van Wijck et al. 1995; Zhang et al. 2000). We have separated the light-dependent degradation of the D1 protein from the synthesis and assembly of the new D1 protein into active

PSII complexes using three experimental systems: (i) studies of photoinhibition and recovery from light stress with intact cells in the presence or absence of chloramphenicol; (ii) synthesis of D1 protein using an *in organelle* translation system; and (iii) sucrose gradient fractionation of chloroplast pigment–protein complexes to study PSII assembly.

Materials and methods

Cell suspension culture conditions

Two different photosynthetic cell suspension cultures from soybean (*Glycine max* L. cv. Corsoy) were used in this study. The SB-P line (Rogers et al. 1987), kindly provided by Prof. J.M. Widholm (Department of Agronomy, University of Illinois at Urbana, USA), was considered here as the WT. The STR7 mutant was obtained from the SB-P line by selection against s-triazine herbicides. Both cell lines were grown at 24°C in KN^1 medium as previously described (Alfonso et al. 1996).

In vivo high-light treatment

For high-light (HL) experiments, 3-week-old WT and STR7 cell suspensions were placed in a thermostatted cuvette (AFORA, Spain) at 24°C. The cell suspensions were stirred during illumination with 1,000 μmol photons m^{-2} s^{-1} white light. At certain times, cell aliquots were taken to monitor variations in the ratio of F_v to F_m. For recovery experiments, HL-treated cells were placed again in the incubator shaker under normal growth conditions. Photoinhibition and recovery experiments were also carried out at temperatures different from those of the normal growth culture. To that end, 3-week old WT and STR7 cell suspensions were placed in the thermostatted cuvette at 17, 21, 24 and 27°C. Cells were stirred during illumination with 750 μmol photons m^{-2} s^{-1} white light. For recovery experiments, cells were placed in a similar incubator, under normal growth conditions, except that the temperature was maintained at that used for each photoinhibition experiment (17, 21, 24 or 27°C, respectively). In some experiments, chloroplast protein synthesis was inhibited by adding chloramphenicol (Sigma) at a concentration of 300 μg ml^{-1}. Cell suspensions were incubated in the presence of chloramphenicol for 15 min before HL or recovery treatments.

Chlorophyll fluorescence measurements

Chlorophyll (Chl) fluorescence from the photosynthetic cell suspension cultures was measured using a PAM fluorimeter (Walz, Effeltrich, Germany). The ratio of variable fluorescence to maximal fluorescence (F_v/F_m), representing the potential quantum yield of PSII, was measured in dark-adapted cell suspensions. Cells (15 μg Chl ml^{-1}) were stirred in a thermostatted cuvette and dark-adapted for 20 min before F_v/F_m measurements were made. Chlorophyll concentration was determined as described in Alfonso et al. (1996).

Thylakoid isolation and in vitro HL experiments

Isolation of thylakoids from cell suspensions and measurements of photosynthetic activity were carried out as previously described (Alfonso et al. 1996). Photoinhibition of PSII in thylakoids was induced by illuminating thylakoids with 1,000 μmol photons m^{-2} s^{-1} white light from a projector lamp. Thylakoids (100 μg Chl ml^{-1}) were resuspended in reaction buffer (0.3 M sucrose, 10 mM NaCl, 25 mM Mes–NaOH, pH 6.5) without 2,6-dichloro*p*-benzoquinone (DCBQ) in a thermostatted cuvette at 24°C. Aliquots of thylakoid samples were taken from the cuvette at various time intervals and used for measurements of PSII electron transport activity immediately.

Immunological detection of specific PSII proteins

Thylakoids were isolated after the different experimental treatments. Samples were denatured at room temperature in sample buffer containing 50 mM Tris–HCl (pH 7.2), 2% (w/v) SDS, 2 M urea, and 40 mM dithiothreitol (DTT). SDS–PAGE of thylakoid proteins from WT and STR7 was carried out in a 15% acrylamide gel containing 6 M urea in both resolving and stacking gels. Protein concentration was determined using the Bio-Rad protein assay kit. After electrophoresis, the polypeptides were electroblotted and transferred onto nitrocellulose membranes with a Bio-Rad apparatus and probed with antiserum against the specific PSII proteins. The antibody used to detect the D1 protein was raised against a synthetic peptide homologous to the N-terminus (a kind gift from Dr. Autar K. Matoo, Plant Molecular Biology Laboratory, USDA/Agricultural Research Service, Beltsville, MD). Antibodies used to detect the LHCII complex and the 33-kDa extrinsic protein were raised in rabbit against these proteins from spinach (kindly provided by Dr. Matilde Barón, Estación Experimental del Zaidín, Granada, Spain). Antibodies against the D2 protein from spinach were a kind gift from Dr. A. Trebst (Germany). Bands were quantified by densitometry using the NIH image software (available at http://www.ncbi.nih.gov).

Isolation of intact chloroplasts and *in organelle* translation

Isolation of intact chloroplasts on Percoll gradients was carried out essentially as described in van Wijck et al. (1995). *In organelle* translation of freshly isolated intact chloroplasts was performed essentially as described in van Wijck et al. (1995) with the modifications of Zhang et al. (1999). Chloroplasts (0.5 mg Chl ml^{-1}) in Medium C [330 mM sorbitol, 50 mM Hepes–KOH (pH 8.0), 10 mM DTT] were pre-incubated for 10 min at 23°C under illumination (70 μmol photons m^{-2} s^{-1}) in a translation mixture containing 10 mM ATP and 40 μM of each amino acid except methionine. All solutions contained a cocktail of protease inhibitors: antipain (2 μg ml^{-1}), leupeptin (2 μg ml^{-1}), and Pefabloc (100 μg ml^{-1}). Addition of all of these protease inhibitors was crucial since in the presence of Pefabloc alone several D1 degradation products could still be detected (unpublished observations). After this pre-incubation, carrier-free ^{35}S-labeled methionine (Amersham) was added to a final concentration of 18.5 kBq μl^{-1}. Chloroplasts were pulse-labeled for 2.5 min followed by an additional 5-, 10-, or 15-min chase in the presence of 10 mM cold methionine under low light (LL; 70 μmol photons m^{-2} s^{-1}) or HL (750 μmol photons m^{-2} s^{-1}) conditions. Translation was stopped by adding 10-fold volume of ice-cold Medium B [330 mM sorbitol, 5 mM ascorbate, 2 mM EDTA, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 50 mM Hepes–KOH (pH 7.6)]. Fractions were analyzed by SDS–PAGE using a 12–22% (w/v) acrylamide gradient including 4 M urea. After electrophoresis, gels were stained and dried. Quantitation of the relative amounts of [^{35}S]methionine-labeled proteins was carried out by scanning and analysis of radioactivity using a Bio-Rad FX molecular imager.

Immunoprecipitation

Immunoprecipitation of PSII proteins was performed essentially as described in Zhang et al. (1999) with several modifications. After pulse–chase labeling, chloroplasts (1 mg Chl ml^{-1}) were solubilized in the presence of 10 mM Tricine (pH 7.8), 10 mM DTT and 2% (w/v) *n*-dodecyl-β-D-maltoside (DM) in the dark at 4°C for 55 min with occasional mixing. Samples were diluted with 4 vol. of 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1% (w/v) Triton X-100. Saturating amounts of D1 antibody were added to the sample and incubated at 22°C overnight. The mixture was then added to a BSA-saturated Protein A–Sepharose CL-4B slurry (Amersham–Pharmacia) and incubated again for 10–12 h. Then, the Sepharose slurry was washed five times with 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 1% (w/v) Triton X-100. A final wash with 50 mM Tris–HCl (pH 7.5) was applied. The bound antigen was released with 50 mM Tris–HCl

buffer (pH 7.5) containing 1% (w/v) SDS. Immunoprecipitated proteins were separated by SDS–PAGE. Gels were dried and subjected to radiolabeling detection using a Bio-Rad FX molecular imager.

Sucrose gradient analysis of PSII fractions

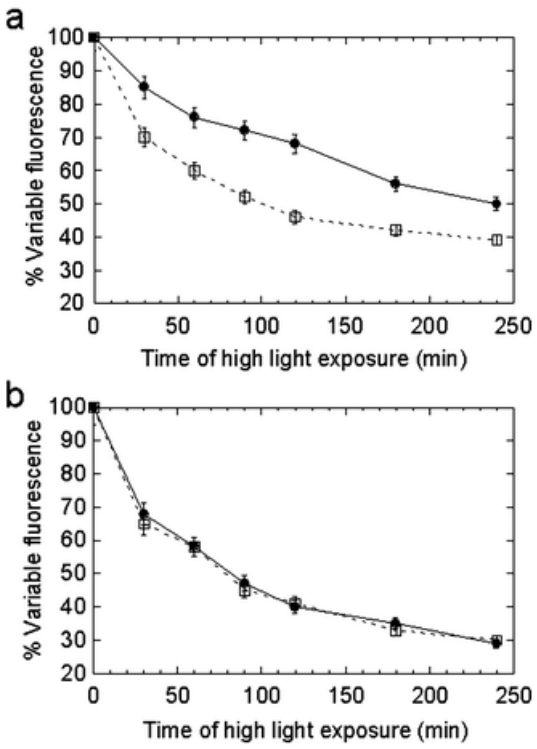
Assembly of PSII fractions was analyzed by sucrose gradient fractionation. Intact chloroplasts isolated from LL-treated cells were resuspended at 1 mg Chl ml^{-1} in 10 mM Tricine buffer (pH 7.8). Then, 1 vol. of 10 mM Tricine buffer (pH 7.8) containing 2% (w/v) DM was added and chloroplasts were solubilized in the dark at 4°C for 55 min with occasional mixing. Subsequently, the suspension was loaded on a 12-ml (0.1–1 M sucrose) linear sucrose gradient containing 0.05% (w/v) DM and centrifuged at 180,000 *g* for 22 h in an SW41.Ti rotor. After centrifugation, the gradient was fractionated into 10 fractions of equal volume from the top to the bottom. The absorption spectrum and total Chl for each fraction were determined before freezing. Proteins were separated by SDS–PAGE using a 12–22% (w/v) acrylamide gradient. Gels were stained with Coomassie Brilliant Blue, dried and subjected to densitometric analysis of the bands using a Bio-Rad FX molecular imager. Replica gels were subjected to immunoblot with antibodies against specific PSII proteins.

Results

Photoinhibition and recovery in WT and STR7 cell suspensions

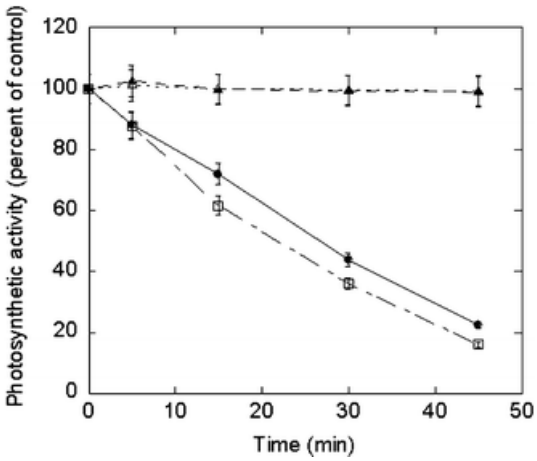
The in vivo inactivation of photosynthesis in WT and STR7 cell suspensions after exposure to HL (1,000 μmol photons m^{-2} s^{-1}) is shown in Fig. 1. Both cell lines showed a decline in F_v/F_m with time, though the STR7 mutant was more sensitive than the WT (Fig. 1a). The STR7 cell suspension lost 50% of variable fluorescence after 90 min of exposure to HL while the WT needed nearly 250 min to reach a similar level of inactivation. In the presence of chloramphenicol, an inhibitor of chloroplast protein synthesis, no significant differences in the extent of photo-inactivation between WT and STR7 cells were observed (Fig. 1b). Both cell lines lost 50% of F_v/F_m after 80 min of photoinhibition. Under these conditions, the D1 repair mechanism is inactivated. Since the data in the presence of chloramphenicol reflect the kinetics of PSII damage in the absence of D1 synthesis and re-assembly of functional PSII, our data suggest that the increased sensitivity to

photoinhibition detected in STR7 compared to WT is more related to protein synthesis and re-assembly rather than the rate of photo-degradation.



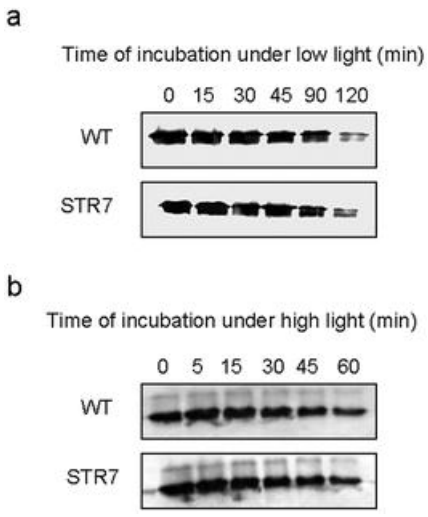
In vitro photoinhibition in isolated thylakoid membranes

To uncover any differences between WT and STR7, we carried out photoinhibition studies using thylakoid membranes (where the recovery machinery is absent). Thylakoids from WT and STR7 were photoinhibited at 24°C under a light intensity of 1,000 μmol photons m^{-2} s^{-1}, and the photosynthetic activity, measured as oxygen evolution in the presence of DCBQ as artificial electron acceptor, was determined. As expected, photoinhibition occurred more rapidly in isolated thylakoid membranes than in intact cells from both cell lines (Fig. 2). However, no significant differences were found in thylakoids from WT and STR7 in contradiction of what occurred in whole cells in the absence of chloramphenicol. Dark-incubated control samples maintained stable activity (Fig. 2). The results with thylakoids are similar to those obtained from in vivo photoinhibition in the presence of chloramphenicol, and strongly suggest that the rate of D1 protein photo-damage is indistinguishable in both cell lines.



Light-induced degradation of the D1 protein in STR7

The light-induced degradation profiles of the D1 protein from both WT and STR7 cells were determined under two different light conditions: 70 μmol photons m^{-2} s^{-1} (LL) and 1,000 μmol photons m^{-2} s^{-1} (HL). Chloramphenicol was added in order to measure only the light-induced D1 degradation. After exposure to LL, the amount of D1 protein decreased in both cell lines; however, no major differences in D1 protein degradation rates were detected between WT and STR7 (Fig. 3a). After 90 min of illumination, the D1 protein content fell by 50% in both cases. When the cells were illuminated with HL, the D1 protein degradation rate was accelerated in both cell lines (Fig. 3b) when compared with LL (Fig. 3a). The half-life of the D1 protein under HL conditions in the presence of chloramphenicol was around 30–45 min for both cell lines (Fig. 3b). Thus, the light-induced D1 protein degradation rates were similar in WT and STR7 cell suspensions under both LL and HL conditions.



Recovery from photoinhibition at normal growth temperature

We studied the recovery of photosynthetic activity from WT and STR7 cells after exposure to HL, since this process is influenced directly by D1 synthesis and assembly. WT and STR7 cells were exposed to HL (1,000 μmol photons m^{-2} s^{-1}) at 24°C till approximately 40–50% of total F_v was lost. After photoinhibition, cells were incubated under normal growth conditions (LL; 70 μmol photons m^{-2} s^{-1}) or in the dark, and the recovery of photosynthetic activity was monitored (Fig. 4). WT cells regained almost 100% of the original F_v/F_m (40% increase) after 120 min of recovery. However, under the same experimental conditions, STR7 cells recovered only up to 76% of the original activity (a 12–16% increase). Almost no recovery was observed for either cell line in the dark (Fig. 4) or in the presence of chloramphenicol during illumination (data not shown). These data confirmed our previous hypothesis that the recovery from photo-inactivation was modified in the STR7 mutant compared to the WT.

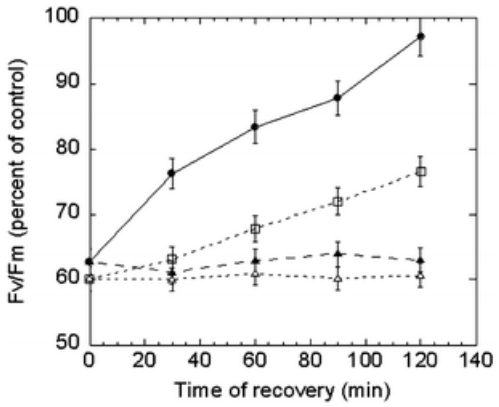


Fig. 4 Recovery of PSII activity after photoinhibition in suspension-cultured WT and STR7 cells of soybean. The cells were photoinhibited (1,000 μmol photons m^{-2} s^{-1}) at 25°C till 40% of the initial fluorescence values were lost (reversible phase). Then, half of the culture was transferred to normal growth illumination (70 μmol photons m^{-2} s^{-1}) at 25°C. The rest was kept in the dark under the same temperature conditions. F_v/F_m values are shown for WT under LL (●), STR7 mutant under LL (□), WT in darkness (▲), STR7 in darkness (△). Initial values of F_v/F_m in WT and STR7 cells were 0.78 and 0.73, respectively. Values represent means ± SE of three independent experiments

Photoinhibition and recovery as a function of temperature

Photo-damage to D1 and the resulting photo-inactivation of PSII is substantially independent of temperature (Tyystjärvi and Aro 1996). By contrast, the process of D1 replacement is highly temperature dependent (Gombos et al. 1994). We studied the temperature dependence of

photoinhibition (considered as the resultant balance between photo-inactivation and recovery) and recovery in both the WT and STR7. As shown in Fig. 5, we first compared the kinetics of F_v/F_m decline under HL (750 μmol photons m^{-2} s^{-1}) at several temperatures near the normal growth temperature (24°C) of cell suspensions. At 24°C, STR7 showed a higher sensitivity to light than the WT, confirming the results of Fig. 1 (top right panel). At 27°C, the differences between the two cell lines became much smaller and both cell lines lost approximately 50% of control F_v/F_m values after 4 h of photoinhibition (Fig. 5, top left panel). Interestingly, at this temperature the decline rate of STR7 remained in fact very similar to that at 24°C but that from the WT became significantly faster. At 21°C, the F_v/F_m ratio declined much faster than at normal growth temperature in STR7, while little difference was found in the decline of the WT when compared to its own decline at 24°C (Fig. 5, bottom left panel). In fact, the biggest differences between the two cell lines were found at 21°C. At 17°C the F_v/F_m ratio declined in both cell lines. Again, the STR7 mutant was more sensitive to photoinhibition than the WT; STR7 cells lost 50% of the initial F_v/F_m values in less than 2 h of HL, while it was necessary to treat WT cells for 3 h to reach the same value (Fig. 5, bottom right panel). It is interesting that at 17°C, the F_v/F_m ratio declined significantly faster in the WT compared to 21°C while the differences were less apparent in the STR7 at both temperatures. It is also worth mentioning that when the temperature was decreased to 17°C the rate of photoinhibition for the WT was nearly identical to that obtained for STR7 at normal growth temperature (24°C).

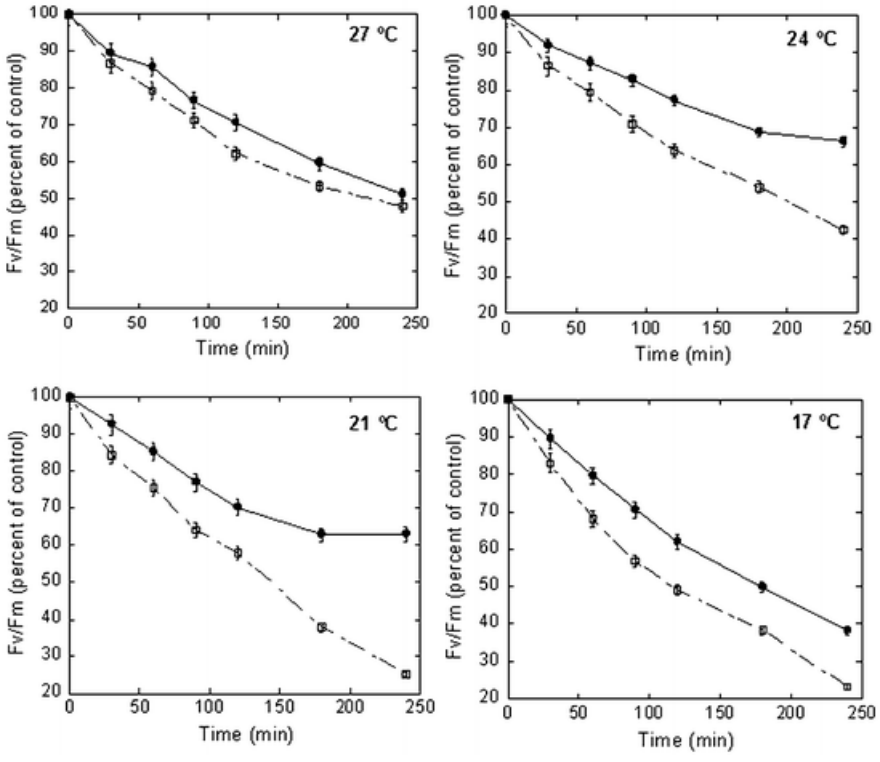


Fig. 5 Photoinhibition in suspension-cultured cells of the WT (●) and STR7 mutant (□) of soybean at different temperatures. Cells were grown at LL (70 μmol photons m^{-2} s^{-1}) and then transferred to HL (750 μmol photons m^{-2} s^{-1}) at 27°C (*top left panel*), 24°C (*top right panel*), 21°C (*bottom left panel*) or 17°C (*bottom right panel*). Variable fluorescence was monitored at different times. Initial values of F_v/F_m in WT and STR7 cells were 0.78 and 0.73, respectively. Data represent means ± SE of three independent experiments

We next studied the temperature effect on the recovery from photoinhibition. To that end, cells were photoinhibited at different temperatures as described above (i.e., 27, 24, 21 and 17°C) till they lost approximately 40% of the initial F_v/F_m values, in order to avoid any irreversible damage that could affect the recovery process. After photoinhibition, the suspensions were placed at the different temperatures under LL (normal growth light; 70 μmol photons m^{-2} s^{-1}) and the changes of the F_v/F_m ratio with time were monitored (Fig. 6). At 24°C (normal growth temperature), the WT cells recovered much faster than the STR7 cells, i.e., in WT the recovery was almost completed after 125 min of incubation but in the case of STR7 the recovery was only around 75% after that time (Fig. 6, top right panel). At 27°C, the recovery rate in the STR7 cells was essentially the same as that at 24°C but in the case of WT the recovery was much slower (Fig. 6, top left panel). As a consequence, both cell lines recovered up to 85–90% after 180 min incubation. These results are consistent with the photoinhibition rates obtained in Fig. 5, i.e., temperatures higher than that of normal growth have stronger negative effects on WT than on STR7 cells. Again, temperatures lower than that of normal growth (24°C) had dramatic effects on the STR7 cells. At 21°C, the recovery rate of the STR7 mutant was highly affected while the WT was also affected but at much lower level (Fig. 6, bottom left panel). As a consequence, the recovery of STR7 was almost negligible after 3 h (less than 10%) and the difference between the two cell lines was even higher at 21°C than that at normal growth temperature. At 17°C, the recovery of STR7 cells was again negligible (less than 5% after 3 h incubation) but the recovery of the WT cells was also significantly reduced (only 15%). As occurred with the photoinhibition rates, when temperature was decreased to 17°C, the recovery rate of the WT was almost identical to that obtained in STR7 at normal growth temperature (24°C).

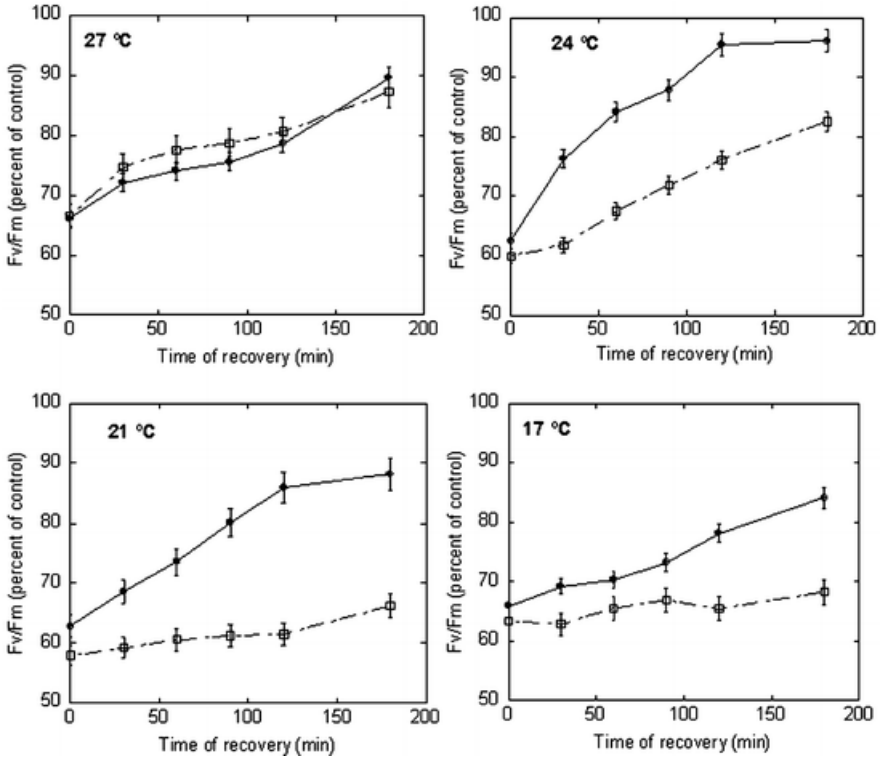


Fig. 6 Temperature dependence of the recovery from photoinhibition in WT (●) and STR7 mutant (□) cells of soybean. The cells were photoinhibited (750 μmol photons m^{-2} s^{-1}) at 27, 24, 21 or 17°C till 40% of the initial fluorescence values were lost (reversible phase). Then, the culture was transferred to normal growth illumination (70 μmol photons m^{-2} s^{-1}) at 27°C (*top left panel*), 24°C (*top right panel*), 21°C (*bottom left panel*) or 17°C (*bottom right panel*). Samples were taken to monitor the recovery of the F_v/F_m ratio. Initial values of F_v/F_m in WT and STR7 cells were 0.78 and 0.73, respectively. Values represent means ± SE of three independent experiments

De novo synthesis of D1 protein and its incorporation into thylakoid membranes

In the light of the previous data, we studied in detail the turnover mechanism of the D1 protein in both cell lines. Two steps should be differentiated in this process: (i) D1 protein synthesis itself, comprising D1 synthesis initiation, elongation and maturation; and (ii) the incorporation of the newly synthesized D1 protein into the membrane and its assembly in PSII complexes. We first carried out [^{35}S]methionine pulse–chase experiments under LL conditions to determine the amount of newly synthesized D1 translation products in both cell lines. The elongating D1 protein in intact chloroplasts from WT and STR7 was chased in the presence of unlabeled methionine. Figure 7a shows an autoradiogram of the [^{35}S]methionine-labeled newly synthesized translation products in a pulse–chase experiment in intact chloroplasts from WT and STR7. Some radiolabeled bands of

molecular mass greater than the D1 protein were present during the chase (Fig. 7a). They possibly represent tRNA–D1 nascent chain complexes that remained associated with the membranes during the isolation of the preparations (Zhang et al. 2000). Nevertheless, they disappeared during the chase with a concomitant accumulation of full-length D1 protein (Fig. 7a). The D1 protein was the most abundant labeled PSII protein, in agreement with earlier observations in vivo (Matoo et al. 1981; van Wijck et al. 1995; Zhang et al. 2000). The amount of labeled mature D1 protein increased in both cell lines with the time of illumination. However, no significant differences were detected in the amount of D1 protein at the different chase times between the two cell lines (Fig. 7a). Only small amounts, if any, of precursor D1 protein (pD1) were detected. This is mainly due to the fact that processing occurs very fast (3–5 min) in higher-plant chloroplasts (van Wijck et al. 1995). The data indicated that synthesis and processing of D1 was not a limiting step in D1 turnover in STR7.

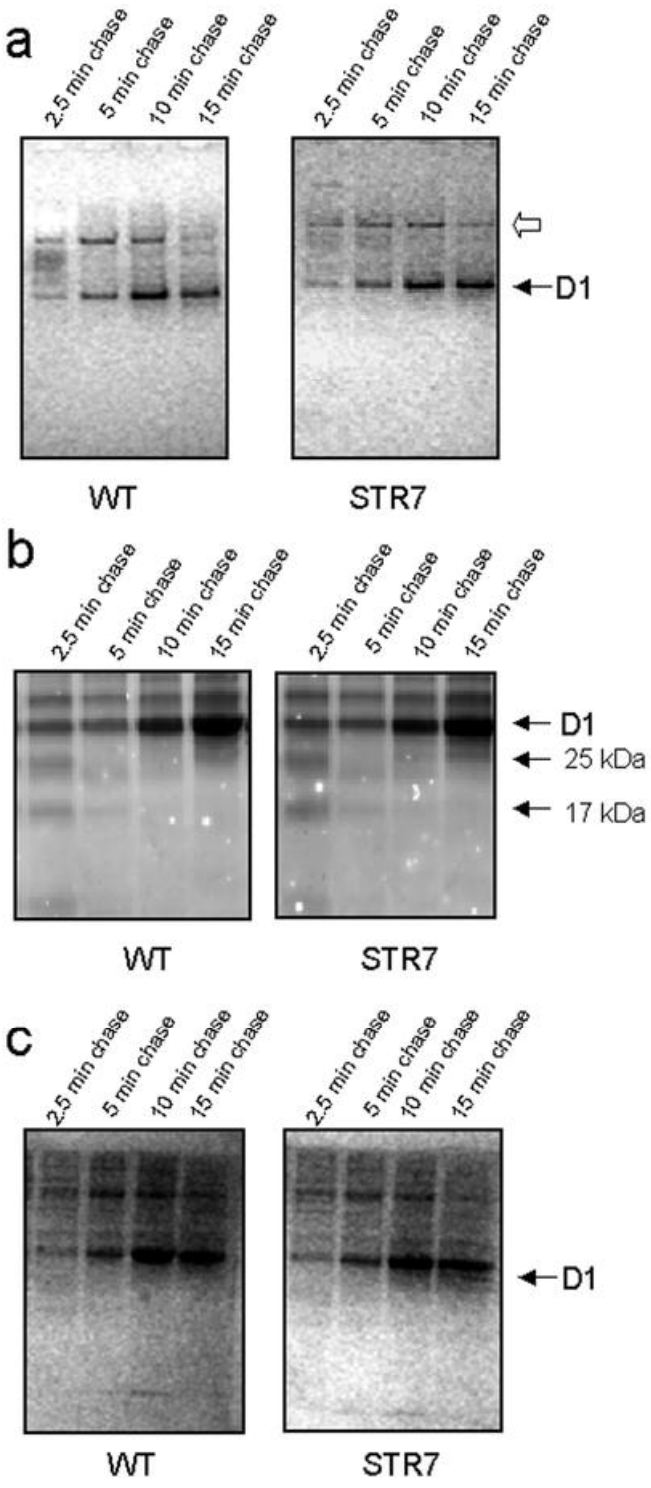


Fig. 7a–c D1 protein synthesis in suspension-cultured cells of the WT and STR7 mutant of soybean. **a** Autoradiogram of the ^{35}S-labeled newly synthesized translation products in a pulse–chase experiment in intact chloroplasts from WT and STR7 under LL conditions. Proteins were separated by SDS–PAGE in a 12–22% (w/v) acrylamide gradient gel including 4 M urea. Gels were dried, autoradiographed and labeled proteins were detected with a phosphorimager. The *open arrow* indicates a possible tRNA–D1 nascent chain complex. **b** Immunoprecipitation of labeled D1 products. After [^{35}S]methionine incorporation, chloroplasts were solubilized and labeled D1 products were inmunoprecipitated with antiserum raised against the

N-terminal of the D1 protein. The precipitated products were purified through a protein A column. The precipitated products were separated by SDS–PAGE. Gels were dried, autoradiographed and labeled proteins were detected with a phosphorimager. **c** Autoradiogram of the ^{35}S-labeled newly synthesized translation products in a pulse–chase experiment in intact chloroplasts from WT and STR7 under HL conditions. All experiments were repeated three times

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D1 intermediates have been reported to accumulate during the D1 elongation process (Zhang et al. 1999, 2000). We carried out a similar experiment in which labeled chloroplasts were solubilized and subjected to immunoprecipitation in the presence of an excess of D1 antibody. Several bands corresponding to labeled proteins were precipitated by the D1 antibody (Fig. 7b). By the end of a 5-min chase, most of the radioactivity had accumulated into full-length D1 protein. The amount of full-length D1 protein accumulated during the different chase periods was very similar in both cell lines, suggesting that no major differences in D1 translation and elongation occur. Two lower-molecular-weight bands of around 25 and 17 kDa were also precipitated by the D1 antibody (Fig. 7b). These bands may well correspond to the 25- and 17-kDa D1 elongation intermediates detected by other groups (Zhang et al. 1999, 2000). Even when these D1 elongation products were slightly more abundant in STR7 after a 2.5-min chase, no differential accumulation was detected during illumination. This suggests that no major differences in the synthesis and elongation of the D1 protein take place in the STR7 mutant under LL conditions compared to the WT.

Since accelerated synthesis of D1 is required during HL illumination, D1 protein synthesis was tested in the mutant and WT cells under HL in order to exclude any difference in the capacity for D1 synthesis as a reason for the enhanced susceptibility of mutant cells to photoinhibition (Fig. 7c). As expected, the amount of radiolabeled methionine incorporated into D1 was higher under HL than under LL conditions, but the rate of D1 synthesis in the two cell lines was very similar (Fig. 7c). The data therefore indicated that D1 synthesis was not related to the enhanced HL sensitivity found in STR7.

Analysis of PSII assembly in WT and STR7 chloroplasts by sucrose gradient centrifugation

We analyzed the assembly of PSII complexes in chloroplasts from both WT and STR7 cell lines. To that end we followed the method described in van Wijck et al. (1995), which consists of the solubilization of chloroplasts from plants grown under LL conditions with the detergent *n*-dodecyl

maltoside (DM) and subsequent sucrose gradient fractionation. We carried out the same experiments with LL-cultured cells. A schematic representation of the band profile obtained from a typical sucrose gradient is shown in Fig. 8a. Six bands, from bottom to top were observed repeatedly and numbered with Roman numerals. According to the fractionation described in van Wijck et al (1995), band VI, which corresponds with the top of the gradient, contained free pigments and proteins (15–30 kDa) in detergent micelles together with the hydrophilic oxygen-evolving extrinsic (OEE) proteins. Band V, corresponding to fraction 2 (40–70 kDa), was mostly composed of minor Chl *a/b* proteins and monomeric LHCII complex. Free D1 and D2 proteins can also be found in band V. Band IV, which correspond to fractions 3 and 4 (80–150 kDa), contained mostly trimeric LHCII complexes and PSII reaction-center particles. Band III, which corresponds to fraction 6 (195–240 kDa), contained PSII cores without the OEE proteins. Band II, corresponding to fractions 7 and 8 (280–360 kDa), contained PSII complexes with bound OEE proteins and also monomeric PSI. Finally, band I, corresponding to fraction 9 (500 kDa and higher), contained mostly PSI in its dimeric form.

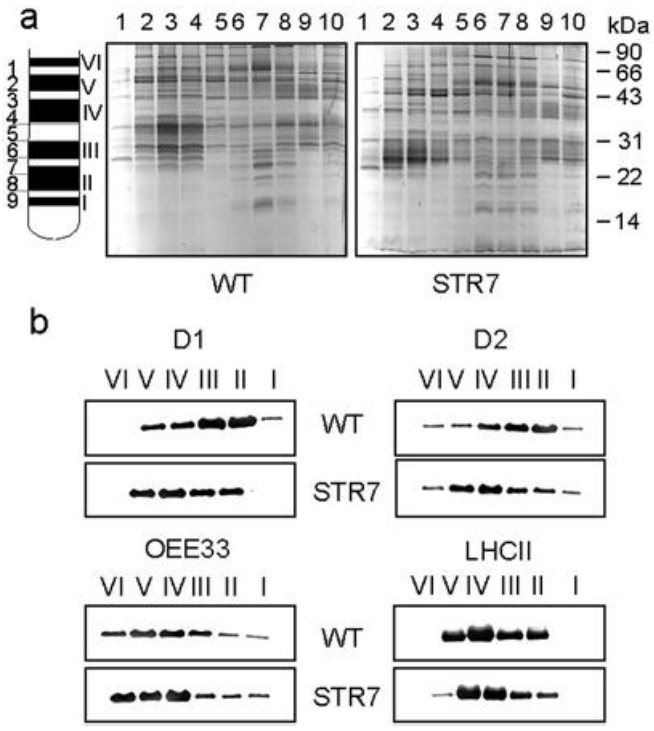

Fig. 8a,b Distribution in sucrose gradients of chloroplast complexes and proteins from suspension-cultured cells of WT and STR7 mutant soybean. **a** Coomassie stain of SDS–PAGE of fractions from sucrose gradients from WT and STR7 chloroplasts. Numbers (1–10) from top to bottom indicate volume fractions while roman numerals (I–VI) indicate position of the bands within the gradient tube. Assignment of the different complexes was determined on the basis of cited literature (Van Wijck et al. 1995). **b** Immunodetection by Western blot of specific PSII proteins in the sucrose gradient bands. Specific antibodies against the PSII proteins D1, D2,

OEE33 and LHCII were used. Representative immunoblots are shown for each specific antibody. All experiments were repeated twice

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A Coomassie Brilliant Blue stained gel of the sucrose gradient protein fractionation is shown in Fig. 8a. The most striking difference detectable at this level is related to proteins in the range 28–25 kDa. These proteins correspond mainly to the LHCII and minor Chl *a/b* antenna complexes. They migrated into higher-density fractions in the WT. The highest amount of LHCII complex was detected in fractions 3 and 4 (Fig. 8a). In contrast, the highest accumulation of LHCII antenna in the STR7 mutant gradient was detected in fractions 2 and 3, while reduced levels of this complex were detected in fraction 4. These results indicated that the LHCII complex migrated in the sucrose gradient into lower-density fractions in the STR7 mutant, suggesting significant differences in its aggregation states. Subsequent pigment analysis of the gradient bands further confirmed these differences (data not shown).

Distribution pattern of D1 and D2 polypeptides

An immunological characterization using specific antibodies against PSII proteins of the different fractions from the sucrose gradient was carried out. We used specific antibodies against the D1 and D2 reaction-center proteins, the LHCII complex and the 33-kDa extrinsic protein (Fig. 8b). In WT and STR7 chloroplasts, the D1 protein was distributed all through the sucrose gradient, the highest accumulation being in bands II and III in WT (Fig. 8b). The results are consistent with those obtained for spinach chloroplasts (van Wijck et al. 1995). Band III contained PSII cores consisting of D1/D2/CP43/CP47/Cytb_{559}/*psbI* in the monomeric form and band II contained PSII complexes mostly in their dimeric form. These results indicated that in the WT most of the D1 protein was detected as assembled complexes with the highest accumulation in the fraction corresponding to the highest degree of assembly of PSII. However, STR7 chloroplasts showed a different pattern of D1 protein accumulation. Although the D1 protein was detected in almost all the fractions, including bands II and III, the biggest differences in the accumulation of the D1 protein with respect to WT were detected in bands IV and V (Fig. 8b). According to the distribution pattern described in van Wijck et al. (1995), band IV contained PSII reaction-center particles (D1/D2/Cytb_{559}) without core antenna complexes (CP43 and CP47). D1 and D2 proteins detectable in band V represent "free" D1 and D2 proteins. The data indicated that after solubilization and fractionation of STR7 chloroplasts, most of the D1 protein was detected in monomeric PSII cores or reaction-center particles rather than in PSII complexes with a greater degree of assembly. These

results were confirmed using antibodies against the D2 protein. D1 interacts with the D2 protein very early during the co-translational insertion process (Zhang et al. 1999). Distribution of the D2 protein in the sucrose gradient from WT chloroplasts was very similar to that observed for the D1 protein (Fig. 8b). The data suggested that in WT, the D1 protein interacted rapidly with D2, forming PSII cores that were routinely assembled into PSII complexes. In contrast, in STR7 the D2 protein accumulated mostly into bands III, IV and V that contained PSII reaction-center particles and cores (Fig. 8b). These results suggested that even if D1 seemed to interact normally with D2 in the mutant, both proteins were less efficient in the PSII assembly process, forming more PSII cores and fewer PSII complexes.

Distribution pattern of the LHCII complex and the OEE 33-kDa protein

We compared the assembly pattern of LHCII and OEE33 with D1 since their mechanisms of synthesis and incorporation to PSII differ (Fig. 8b). In WT chloroplasts, the highest accumulation of LHCII was observed in bands IV and V, with much higher amounts in band IV, suggesting that most of the major antenna complexes detected in WT chloroplasts are in the oligomeric form. In contrast, the distribution pattern of the LHCII complex in STR7 chloroplasts was different, with significant accumulation in band V and reduced levels in band IV (Fig. 8b). The results were consistent with the SDS–PAGE analysis (Fig. 8a), and indicated that in the STR7 chloroplasts most of the LHCII complex was present in the monomeric form, though a portion was in oligomeric form. Fluorescence emission spectra at 77 K of WT and STR7 thylakoids further confirmed this result (data not shown). Our data are significant in the context of PSII assembly and dynamics since oligomerization of the LHCII complex is necessary prior to its association with the PSII core in the grana (Hobe et al. 1994).

The distribution of the OEE33 protein is also shown in Fig. 8b. OEE33 is synthesized as a precursor protein in the cytoplasm and post-translationally targeted across the chloroplast membrane before assembling into the PSII core (Theg and Scott 1993). It has been proposed that the OEE33 protein binds to PSII cores or minimal PSII complexes in the stromal regions and then migrates to the grana (Hashimoto et al. 1997). The distribution of OEE33 in WT chloroplasts was quite homogeneous, with an increased accumulation in bands III, IV and V (Fig. 8b). In STR7, most of the protein accumulated in bands IV and V, as in the case of WT, though significant amounts also accumulated in band VI (Fig. 8b).

Discussion

In this work we have studied light-induced PSII inactivation and recovery from photoinhibition in the STR7 soybean cell line compared to the WT. Special effort was made to identify the step or steps that become rate-limiting and are responsible for the higher light-sensitivity found in STR7 compared to WT. To that end, we studied D1 synthesis and replacement mechanisms in detail in both cell lines. To our knowledge, this is the first time that such a detailed analysis has been carried out in higher plants to study the increased sensitivity to light.

In vivo photoinhibition experiments, carried out under conditions that reflect PSII damage kinetics in the absence of D1 synthesis and repair, demonstrate that PSII photo-inactivation is not involved in the different sensitivities to photoinhibition observed in the two cell lines. They also prove that in STR7 there is no influence of the S268P mutation in the Q_B pocket on the photo-inactivation rate, suggesting that in higher plants, there is no correlation between the structure of D1 and photo-inactivation. This last conclusion is consistent with previous results using several *psbA* mutants from cyanobacteria (Kirilovsky et al. 1989; Nixon et al. 1995; Mulo et al. 1998).

Since photoinhibition as a whole process can be considered as a dynamic balance of PSII photo-inactivation and recovery, we have to conclude that the latter is impaired in the case of the STR7 mutant. This is in fact demonstrated by the recovery experiments of Figs. 4 and 6. The recovery process involves several steps, from de novo synthesis of D1 to the assembly and reconstitution of a fully active PSII complex (van Wijck et al. 1995; Zhang et al. 1999). One or several of these steps were altered in STR7. Our results indicate that the synthesis and elongation of the D1 protein occur at the same rate in the STR7 mutant and the WT whatever the light conditions, whereas the re-assembly of the newly synthesized D1 protein into PSII complexes is altered in STR7 even under growth light (LL) conditions. Interestingly, not only D1 but also other PSII proteins like D2, or the LHC complex also presented modifications in their assembly pattern. Our data indicate that the main alteration was not related to the first steps of the assembly (D1 elongated similarly in both cell lines and D2 accumulation followed that of D1, indicating that the D1/D2 interaction during the elongation process was not substantially altered) but with the latter steps where PSII core particles are associated to form fully active PSII complexes in their dimeric form. Our results suggest that these alterations in PSII assembly may become the rate-limiting step under HL conditions, when D1 turnover is accelerated, and may explain the increased sensitivity to photoinhibition found in STR7.

Several hypotheses could explain the different PSII assembly in the STR7 mutant. One possibility is related to the intrinsic effects of the mutation. Because of the key position of S268, located within the Q_B binding site, at the beginning of the E helix of D1 (Zouni et al. 2001), S268 may contribute to the overall stability of the D1 protein structure. A mutation in this residue might introduce some conformational changes in D1 that may render the mutated reaction centers less efficient to function as an acceptor complex during PSII assembly (van Wijck et al. 1995; Zhang and Aro 2002). On the other hand, we must consider that the recovery process is a complex dynamic phenomenon where PSII proteins have to be synthesized and inserted into the thylakoid membrane (like D1), and move from the stroma to the grana during re-assembly. Thus, the dynamic properties of the membrane matrix should play an important role in the recovery process. As was mentioned in the Introduction, membrane fatty acid saturation is higher in STR7 than in WT, imposing different physical–chemical characteristics on the membrane. Previous work by our group has demonstrated that this is in fact the case. Using differential scanning calorimetry (Alfonso et al. 2001) and spin probe EPR (Yruela et al. 2001) techniques, it was shown that the thylakoid membrane matrix was more rigid in STR7 than in WT. These changes in membrane fluidity would greatly affect a typically dynamic process such as photoinhibition and recovery. In that sense, the observation that temperature variations around 24°C during photoinhibition and recovery affected the two cell lines in opposite directions, i.e., higher temperatures affected WT recovery more negatively and lower temperatures affected STR7 dramatically, is important. The greatest differences between the two cell lines appeared at 21°C, demonstrating that lowering the temperature only 3°C results in a dramatic effect on STR7 recovery. These data are similar to those reported in *Arabidopsis* mutants deficient in fatty acid unsaturation (Vijayan and Browse 2002). Furthermore, the photoinhibition and recovery rates of the WT at 17°C mimic those of STR7 at 24°C, strongly supporting a role for fatty acid unsaturation/membrane fluidity in the PSII-assembly alterations found in STR7. This difference of 7°C corresponds closely to the difference in the membrane phase transition determined spectroscopically in these two cell lines (Alfonso et al. 2001; Yruela et al. 2001).

Our data point to a central role for fatty acids from membrane lipids in the assembly and dynamics of PSII. In that sense, some in vitro data have demonstrated that 3-*trans*-C16:1 is necessary for trimerization of LHCII (Hobe et al. 1994) and that the presence of PG-containing 3-*trans*-C16:1 is critical for PSII dimerization (Kruse et al. 2000). More recently, it has been shown that 3-*trans*-C16:1 esterified at the sn-2 position of PG, was required for the stabilization of trimeric

LHCII and dimeric PSI under conditions of high biosynthesis rate in *Chlamydomonas* (Dubertret et al. 2002).

With the results presented in this paper, we cannot preclude either of the two hypotheses to explain the light-sensitivity phenotype found in STR7; both could be even operative in the mutant. It is true that any effect of the mutation would be expected to act on the photoinactivation rate of the mutated D1 or during the elongation process and interaction with D2. However, these processes seemed not to be affected in STR7. The following facts may favor the "fatty acid hypothesis": (i) photoinhibition and recovery in STR7 were highly affected in a narrow temperature range around that of the normal growth; (ii) the photoinhibition and recovery rates of WT at 17°C were nearly identical to those for STR7 at growth temperature (24°C); and (iii) not only D1 but other PSII proteins like D2, OEE33 and LHCII exhibited alterations in their assembly pattern in STR7. Future analysis of D1 synthesis and PSII assembly in *Arabidopsis* mutants deficient in fatty acid unsaturation, and herbicide-resistant mutants with mutations other than S268P may help to clarify this point.

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