PsbT Polypeptide Is Required for Efficient Repair of Photodamaged Photosystem II Reaction Center*

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PsbT is a small chloroplast-encoded hydrophobic polypeptide associated with the photosystem II (PSII) core complex. A psbT-deficient mutant (ΔpsbT) of the green alga Chlamydomonas reinhardtii grows photoautotrophically, whereas its growth is significantly impaired in strong light. To understand the photosensitivity of ΔpsbT, we have studied the effect of strong illumination on PSII activity and proteins. It is shown that the level of PSII activity and proteins is reduced in the ΔpsbT more significantly than in wild type under strong light. When recovery of the photodamaged PSII is impaired by a chloroplast protein synthesis inhibitor, the light-induced inactivation and degradation of PSII occur similarly in wild-type and mutant cells. On the contrary, the recovery of PSII activity after partial photoinactivation is remarkably delayed in the ΔpsbT cells, suggesting that PsbT is required for efficient recovery of the photodamaged PSII complex. These results therefore present the first evidence for involvement of this small PSII polypeptide in the recovery process. Partial disintegration of the purified PSII core complex and localization of PSII proteins in the resulting PSII subcore complexes have revealed that PsbT is associated with D1/D2 heterodimer. A possible role of PsbT in the recovery process is discussed.

Light energy is converted into redox energy by two photosystems, photosystem I (PSI) and photosystem II (PSII), and thus is used to drive photosynthetic electron transport from water to NADP⁺. Although light energy is essential for photosynthesis, excess light is harmful to photosynthetic apparatus. Damage to photosynthetic apparatus caused by excess light is designated as photoinhibition and it is generally accepted that the primary target for the photoinhibition is PSII (1–4). It has been proposed that strong light illumination of photosynthetic organisms results in the overreduction of the primary acceptor of PSII, QA to QA⁻H₂ and the subsequent release of QA⁻H₂ from the PSII complex. In light, the PSII complex depleted of QA generates the triplet state of P₆₈₀, which produces a singlet excited oxygen species (¹O₂) in the presence of oxygen. Since ¹O₂ is a very reactive and strongly oxidizing species, it may damage PSII proteins, in particular the D1 protein, as well as PSII cofactors (5). The damaged D1 is degraded and subsequently replaced by a newly synthesized copy. This light-induced rapid turnover of D1 as well as replacement of cofactors are essential for maintaining PSII activity in light (6). In addition to this acceptor side photoinhibition, donor side photoinhibition occurs when the oxygen-evolving complex is inhibited. In this case, strong oxidants that are accumulated on the primary electron donor, P₆₈₀, and/or the secondary electron donor, Yz, are thought to trigger degradation of the D1 protein (1–4).

The PSII complex consists of a number of cofactors and more than 20 subunits. The major redox components involved in the photochemical reaction are present in the two homologous reaction center polypeptides, D1 and D2 (D1/D2 heterodimer), that are encoded by the chloroplast psbA and psbD genes, respectively, in eukaryotes (7). The core antenna complexes, CP47 and CP43, of which the apoproteins are encoded by the chloroplast psbB and psbC genes, respectively, and cytochrome b₅₅₉ coded for by the chloroplast psbE and psbF genes are closely associated with the D1/D2 heterodimer (7). In addition to these polypeptides containing cofactors, the PSII complex contains the luminal extrinsic polypeptides, OEE1, OEE2, and OEE3, encoded by the nuclear psbO, psbP, and psbQ genes, respectively (8), and approximately 10 hydrophobic small polypeptides (9). The three extrinsic polypeptides are known to be involved in O₂ evolution, while the small polypeptides might be required for assembly, stability, and/or optimal function of PSII. However, the specific role of the small polypeptides mostly remains to be elucidated.

The PsbT polypeptide with a molecular mass of 4 kDa is one of the small polypeptides of the PSII core complex and has a putative transmembrane helix (10). This polypeptide is encoded by the chloroplast psbT gene; this gene was tentatively called ycf8 (hypothetical chloroplast open reading frame 8) and was designated as psbS in a previous paper (10). According to the international gene nomenclature, this gene is renamed psbT. The psbT gene is located in a highly conserved gene cluster, psbB-psbT-psbN-psbH genes, in the chloroplast genome (11–16).

In the previous report (10), a psbT deletion mutant (ΔpsbT) has been generated from the green alga Chlamydomonas reinhardtii by particle-gun mediated chloroplast transformation. The resulting mutant was able to grow photoautotrophically like wild type in the moderate light. Since the activity and amount of PSII in the mutant were only slightly impaired as compared with those in wild-type cells, it was concluded that PsbT is not essential for synthesis, stability, and function of the PSII complex. An intriguing characteristic of the ΔpsbT mutant is that the cell growth and PSII activity were significantly impaired under certain adverse growth conditions. Interestingly, no mutant cell growth was observed under light stress.

* This work was supported in part by Grants-in-aid for General Research (C)(2) 11640649 and 13640651 and for Scientific Research on Priority Area (A) 12025223 (to Y. T.) from the Ministry of Education, Science, Sports and Culture. This study was also carried out as a part of “Ground Research Announcement for Space Utilization” promoted by Japan Space Forum. The costs of publication of this article were defrayed in part by the payment of page charges. This article must be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: PS, photosystem; TAP, Tris acetate-phosphate; PAGE, polyacrylamide gel electrophoresis; Chl, chlorophyll.
conditions. This finding suggests that PSII is more photosensitive in the ΔpsbT mutant cells than in wild-type cells.

In this study, we have characterized the activity and stability of PSII during strong illumination as well as the recovery of photodamaged PSII. We show that the steady-state level of PSII activity decreased more significantly in ΔpsbT mutant cells in strong light as compared with wild-type cells. According to the results presented here, PsbT does not play a role in the protection of PSII against photoinhibition but is essential for the efficient recovery of the photodamaged PSII. Since the synthesis of chloroplast-encoded proteins was not affected after strong light illumination, PsbT appears to be involved in a post-translational step in the repair process. The possible role of PsbT in the repair process of the photodamaged PSII is discussed.

MATERIALS AND METHODS

Strains and Growth Conditions—C. reinhardtii wild-type strain 137c and a psbT deletion mutant (ΔpsbT) were used in the present study. The ΔpsbT transfectants were generated by particle-gun mediated chloroplast transformation as described (10). Cells were grown to mid-log phase (4 × 10^6 cells ml^−1) in Tris-acetate-phosphate (TAP) medium at 25 °C in the dim light (10 μmol of photons m^−2 s^−1) (17).

Western Analysis—To study the effects of strong light illumination on PSII activity and proteins, cells from mid-log phase were collected by centrifugation (3,200 g for 10 min at 25 °C) and then resuspended in TAP medium at 10 μg of Chl ml^−1. The cells were exposed to strong illumination (4,000 μmol of photons m^−2 s^−1) or kept in the dark as a control. The rate of PSII photodamage was estimated in the presence of chloramphenicol (200 μg ml^−1), which was added to cultures 10 min prior to experiments. To study the recovery of photodamaged PSII activity, cells were photoinhibited for 15 min and subsequently incubated either in the light (40 μmol of photons m^−2 s^−1) or dark.

O₂ Evolution—O₂ evolution of cells (10 μg of Chl ml^−1) was measured by a Clark-type O₂ electrode using actinic light intensity of 5,000 μmol of photons m^−2 s^−1 (17).

Pulse Labeling of Chloroplast-encoded Proteins—Cells grown to mid-log phase (4 × 10^6 cells ml^−1) were either exposed to the strong light or dark. The cells were exposed to 35S-labeled sodium sulfate (50 μCi ml^−1) for 1 min and separated by urea-SDS-PAGE as described in Ref. 18.

RESULTS

Growth of ΔpsbT Mutant Cells in Strong Light—It has previously been reported by cell growth experiments on solid medium that the psbT-deletion mutant (ΔpsbT) of Chlamydomonas is able to grow photoautotrophically and mixotrophically under moderate light conditions (90 μmol of photons m^−2 s^−1) while the growth of this mutant is significantly impaired by strong light illumination (1,200 μmol of photons m^−2 s^−1) (10). To verify the effect of high irradiance on cell growth more quantitatively, we analyzed the growth rate of wild-type and ΔpsbT cells in liquid TAP medium both in strong light and in darkness.

Cells grown to 4 × 10^6 cells ml^−1 in the dim light (10 μmol of photons m^−2 s^−1) were diluted to 5 × 10^6 cells ml^−1 prior to analysis of cell growth. Fig. 1A shows exponential growth of the wild-type and ΔpsbT cells with a doubling time of 12 h in the dark. However, in strong light (4,000 μmol of photons m^−2 s^−1), the wild-type cells showed a lag-phase of several hours and a subsequent exponential growth with a doubling time of 8 h (Fig. 1B). This lag-phase suggests that cells grown in low light need to become adapted to the strong light condition before they start to propagate. In contrast to the wild-type cells, no increase in the ΔpsbT cell density was observed in strong light.
activity of light-induced O₂ evolution was measured by a Clark-type O₂ electrode in the presence of 200 μg of cells (10 g of Chl ml⁻¹) separated by urea-SDS-PAGE, blotted onto nitrocellulose membranes, and probed with antibodies against PSII or PSI polypeptides.

These results confirm the suppression of growth of ΔpsbT cells by excess light.

**Photoinhibition of PSII in ΔpsbT Cells**—It has been established that PSII is one of the major targets for photoinhibition caused by excess light. To evaluate the photoinhibition of PSII in ΔpsbT cells, we measured O₂ evolution of cells that had been exposed to strong light illumination for various periods of time. Fig. 2A shows that upon strong light illumination, the O₂ evolution of wild-type cells was reduced to about 35% of the original level within 15 min. This initial drop in activity was terminated after a short period and followed by a short lag period. Subsequently the activity was restored to more than 50% of the original level after 4 h of strong light illumination. During prolonged strong light illumination, no further decrease in the activity was observed. This result is consistent with the lag-phase and subsequent logarithmic phase of the wild-type cell growth in strong light as shown in Fig. 1B. The extent of the inactivation in ΔpsbT cells was obviously more significant than that of wild-type cells, although the initial drop of the activity occurred at the same rate as for wild-type cells. It is noted that a slow decrease in the PSII activity continued in the ΔpsbT cells even after the photoinhibition phase. Therefore, it is concluded that the initial rapid photoinactivation of O₂ evolution results from a decrease in the steady-state level of the PSII complex, while the steady-state level of the PSII complex, we analyzed the recovery of O₂ evolution after photoinhibition.

**Rate of PSII Photoinhibition**—The decrease in PSII activity in strong light should be determined by the relative rates of photoinactivation of PSII and repair of the photodamaged PSII complex. Therefore, the enhanced decrease in PSII activity of ΔpsbT under strong light conditions could result from accelerated photoinactivation of PSII and/or decreased repair activity of the photodamaged PSII. In order to investigate these possibilities, we measured the rate of photoinactivation of O₂ evolution in the presence of an inhibitor of chloroplast translation, chloramphenicol, which blocks the repair of photodamaged PSII (see Fig. 4C). Fig. 3 shows that the activity of wild type and ΔpsbT decreased at the same rate with a half-decay time of 12 min. Thus, PsbT does not protect PSII activity against excess light irradiation. Interestingly, the initial photoinactivation of PSII in the absence of chloramphenicol shown in Fig. 2A was comparable to that in the presence of the inhibitor. These results suggest that the repair of photodamaged PSII complex is rather slow in the cells that had been adapted to dim light and the repair process is activated by strong light illumination.

The strong light-induced decrease in PSII proteins in the ΔpsbT as shown in Fig. 2B could be due to an accelerated degradation during the illumination and/or a decreased efficiency of repair of the photodamaged PSII with newly synthesized proteins. Therefore, the rate of photodegradation of PSII proteins was measured in the absence of chloramphenicol. Fig. 3, B and C, show that the whole PSII complex was photodegraded in a similar manner in both strains while the amount of PSI polypeptide was constant. In the dark, PSII proteins remained constant during the experimental period (data not shown).

The rates of photodegradation of PSII proteins were estimated by scanning the signals with a densitometer and are summarized in the legend of Fig. 3. It was revealed that no difference in the photodegradation rates of the D1, D2, CP43, and CP47 proteins was observed between wild-type and ΔpsbT cells. The photodegradation of D1/D2 heterodimer (t₁/₂ = 15 min) occurred immediately after the photoinactivation of O₂ evolution (t₁/₂ = 12 min). The photodegradation of CP43 and CP47 was much slower (t₁/₂ = 25 min). Thus, the absence of PsbT does not affect photodegradation of the PSII complex. Interestingly, the half-decay time of PsbT during strong light illumination was estimated to be 17 min, which is only slightly slower than that of D1 and D2. Since the primary target for the photodegradation is D1, it can be concluded that D2 is highly unstable in the absence of D1 probably because of the formation of the D1/D2 heterodimer. PsbT is also rather unstable under the photoinhibition condition, suggesting a close interaction of this polypeptide with the D1/D2 heterodimer.

**Recovery of PSII Activity from Photoinhibition**—The observations shown in Fig. 3 suggest that PsbT could be involved in the recovery of photodamaged PSII complex rather than in the photo-protection of the PSII complex. To confirm this possibility, we analyzed the recovery of O₂ evolution after photoinhibition.
light-induced inactivation of O₂ evolution in wild-type and ΔpsbT cells was inhibited by about one-half of the original level by illuminating the cells with strong light (4,000 μmol of photons m⁻² s⁻¹) for 15 min. Subsequently, the cells were incubated either in the dark or in low light (40 μmol photons m⁻² s⁻¹) to measure the recovery of photodamaged PSII activity.

Fig. 4A shows that the photodamaged activity of wild-type cells was recovered to the initial level after 60 min of the low light illumination while recovery of the activity in ΔpsbT cells was approximately four times slower. The recovery of PSII activity was also observed in the dark although at a very low rate. To recover to the initial level in wild-type cells, about 4 h was needed. The recovery in ΔpsbT cells in the dark was also delayed to a similar extent as compared with the wild-type cells (Fig. 4B), indicating that the slower recovery in ΔpsbT in low light was not due to a low-light induced photoinhibition in this photosensitive mutant. These observations indicate that PsbT is required for the efficient recovery of photodamaged PSII. Fig. 4C shows that no recovery was observed in wild-type and ΔpsbT cells in the presence of chloramphenicol. As already shown (23), the recovery after photoinhibition requires de novo protein synthesis in the chloroplast.

**Synthesis of D1 after Photoinhibition**—It is well known that D1 undergoes a rapid turnover during the photoinhibition-repair cycle. Thus, the slower repair of the photosynthetically active PSII complex in ΔpsbT cells could be ascribed either to a partial photoinhibition of chloroplast-encoded protein synthesis, particularly D1, or to a less efficient post-translational event such as replacement of the photodamaged D1 by a newly synthesized copy. To study D1 synthesis during the recovery after photoinhibition, pulse labeling experiments were carried out. After cells were incubated either in the dark or in strong light (4,000 μmol of photons m⁻² s⁻¹) for 15 min, pulse labeling was carried out with [³⁵S]sulfate in low light (40 μmol of photons m⁻² s⁻¹) in the presence of an inhibitor of nuclear-encoded protein synthesis, cycloheximide. The presence of cycloheximide does not affect the recovery of photodamaged PSII (23).

Fig. 5 shows that major PSII proteins such as D1, D2, CP43, and CP47 as well as the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RbcL) were clearly labeled in low light (40 μmol of photons m⁻² s⁻¹) for 15 min. As pulse labeling was carried out under conditions that inhibit de novo protein synthesis, the labeling was due to a pool of pre-synthesized proteins. To study D1 synthesis during the recovery after photoinhibition, pulse labeling experiments were carried out. After cells were incubated either in the dark or in strong light (4,000 μmol of photons m⁻² s⁻¹) for 15 min, pulse labeling was carried out with [³⁵S]sulfate in low light (40 μmol of photons m⁻² s⁻¹) in the presence of an inhibitor of nuclear-encoded protein synthesis, cycloheximide. The presence of cycloheximide does not affect the recovery of photodamaged PSII (23).

Localization of PsbT within PSII Core Complex—It has already been demonstrated that PsbT is associated with the PSII core complex that contains the D1/D2 heterodimer, CP43, CP47, and several small polypeptides (10). However, the exact location of this polypeptide within the core complex is poorly understood. To investigate the localization of PsbT within the complex, purified PSII core complex was disintegrated into subcore complexes with a chaotropic reagent, potassium thiocyanate (KSCN) as described under “Materials and Methods.” The resulting PSII subcore complexes were subsequently frac-

![Fig. 3](http://www.jbc.org/)

**Fig. 3. Kinetics of inactivation of PSII activity and degradation of PSII proteins during the strong light illumination.** The cells (10 μg of Chl ml⁻¹) were illuminated at 4,000 μmol of photons m⁻² s⁻¹ at 25 °C in the presence of chloramphenicol (200 μg ml⁻¹). A, light-induced inactivation of O₂ evolution in wild-type and ΔpsbT cells. The activity was measured as described in the legend to Fig. 2. B, light-induced degradation of PSII proteins. PSI and PSII proteins were detected by Western analysis. To estimate the amount of the proteins during the illumination, a dilution series (50, 25, and 12.5%) of total cell proteins from non-illuminated cells was also analyzed for a calibration standard. Relative amounts of proteins were estimated by scanning the signals with a densitometer. The half-decay times of D1, D2, CP47, and CP43 were estimated to be 15, 15, 25, and 25 min, respectively, in wild-type and mutant cells. The half-decay time of PsbT in wild-type cells was estimated to be 17 min.
tionated into three distinct fractions (I, II, and III) by DEAE ion-exchange column chromatography, as shown in Fig. 6. Fractions I and II contain core antenna complexes, CP43 and CP47, respectively, and fraction III was mainly composed of the D1/D2 heterodimer. Under this disintegration condition, CP43 and CP47 were almost completely dissociated from the PSII reaction center complex (Fig. 6B). It is noted that approximately two-thirds of the PsbT was separated in fraction III, indicating its co-purification with the D1/D2 heterodimer. The remaining one-third of PsbT was separated at low concentration of NaCl (10 mM). Incubation of the PSII core complex in the buffer containing 0.05% DM but not KSCN at 25 °C resulted in only a partial disintegration of CP43 and CP47 from the core complex (data not shown). In this case, neither release of PsbT nor its co-purification with the partially dissociated CP43 or CP47 was observed. PsbT was only detected in the fraction containing both non-dissociated PSII core complex and subcore complexes that were depleted of either CP43 or CP47. It is therefore concluded that all PsbT is originally located in D1/D2 heterodimer.

DISCUSSION

PsbT Is a New Component of PSII Reaction Center—It has already been observed that PsbT is co-purified with PSII core complex in Chlamydomonas (10) and is present in a PSII subcore complex depleted of CP43 in spinach (24). In the present study, partial disintegration of the PSII core complex and subsequent fractionation has shown that PsbT is associated with the D1/D2 heterodimer, the PSII reaction center. Small polypeptides so far detected in the PSII reaction center are cytochrome b$_{597}$, PsbI, and PsbW (7, 25, 26). Thus it can be concluded that PsbT is a new component of the PSII reaction center.

According to a current model of the three-dimensional structure of transmembrane helices of PSII core complex obtained by electron crystallography on CP43-depleted non-oxygen evolving PSII complex (27) and by x-ray crystallography on oxygen-evolving PSII complex (28), the D1/D2 heterodimer is
located between CP43 and CP47. In addition to 22 transmembrane helices of the D1/D2 heterodimer, CP43, and CP47, seven helices are observed in the proximity to the D1/D2 heterodimer of the CP43-depleted PSII complex (27). They probably correspond to the small hydrophobic polypeptides with a single transmembrane helix such as PsbE, PsbF, PsbI, PsbK, PsbL, PsbT, and PsbW. In the oxygen-evolving PSII complex, more helices which may correspond to small polypeptides were observed in the proximity of the D1/D2 heterodimer and core antenna complexes (28). However, it remains unclear whether PsbT has a direct interaction with D1. Although the psbT gene is co-expressed with the psbB gene (15, 29), no biochemical evidence for functional and/or structural interaction between PsbT and CP47 was obtained in the present study. Improved resolution of the three-dimensional structure of the PSII complex might demonstrate a possible interaction between PsbT and CP47.

In Chlamydomonas, stable accumulation of the PSII complex is affected in mutants that are deficient in one of the small polypeptides associated with D1/D2 heterodimer. Mutants deficient in cytochrome b$_{559}$ accumulate no detectable amount of PSII complex (30), whereas psbI-deficient mutants accumulate PSII complex only to 20–30% of the wild-type level (31). Probably the absence of these polypeptides affects stability and/or synthesis of the PSII complex. In contrast to these mutants, the content and activity of the PSII complex is reduced by only ~10% in the ΔpsbT mutant (10). The difference of the phenotype among these mutants may reflect different localization of the small polypeptides in and/or binding mode to the D1/D2 heterodimer.

Photodegradation of PsbT was rather fast compared with that of CP43 and CP47 and only slightly slower than that of D1 in the presence of chloramphenicol (Fig. 3B). The photodegradation of the whole PSII complex may largely be due to a pleiotropic effect; when D1 is primarily photodegraded and is not replaced by a newly synthesized copy, the whole PSII complex becomes unstable. D2 appears to be rather unstable in the absence of D1 because D2 was photodegraded as rapidly as D1 as shown in Fig. 3. In fact, no detectable amount of D2 was observed in a psbA deletion mutant, Fud7, that completely lacks D1 (32) (data not shown). The marked instability of PsbT during photoinhibition compared with CP43 and CP47 may result from its close association with D1. Prolonged illumination of wild-type cells with strong light resulted in a gradual decrease in the steady-state level of PsbT although the other PSII proteins remained constant (Fig. 2B). This observation suggests that photodegraded PsbT is not replaced by a newly synthesized copy efficiently enough, probably because synthesis of PsbT might be slower than that of D1. It is noted that the nuclear-encoded PsbW is also as unstable as D1 during photoinhibition in the isolated thylakoid (33). However, it is not known whether the steady-state level of PsbW is reduced in vivo during strong light illumination.

PsbT Is Required for Efficient Repair of Photodamaged PSII—We have shown that strong light illumination results in significantly reduced steady-state levels both of PSII activity and proteins in ΔpsbT mutant cells compared with wild-type cells. In the absence of PsbT, the rates of photoinactivation and photoreactivation of PSII complex were not affected, while recovery of photoinhibited PSII activity was remarkably delayed. Thus PsbT is essential for efficient recovery of the photodamaged PSII complex. The inefficient recovery of the photodamaged PSII leads to reduced steady-state levels of PSII activity and proteins and probably a defect in cell growth in strong light. Thus this is the first evidence that one of the small hydrophobic polypeptides of PSII is involved in the repair process of the photodamaged PSII.

The repair of the photodamaged PSII activity is a multistep process. This includes replacement of photodamaged D1 and other PSII proteins by newly synthesized copies, and integration and reactivation of cofactors that were lost during the replacement of the photodamaged PSII proteins. It is known that the synthesis of D1 occurs in the stroma-exposed thylakoids, while the functional complex is preferentially located in the grana thylakoids (34). Thus the repair process may require a lateral translocation of the PSII complex between the grana and stroma-exposed thylakoids. As shown in Fig. 5, strong light illumination enhanced the protein synthesis activity in both wild-type and ΔpsbT mutant strains to a similar extent. It is therefore concluded that the synthesis of chloroplast-encoded proteins was not affected by photoinhibition in the absence of PsbT. Thus we can conclude that a post-translational event(s) of the repair process is impaired in the absence of PsbT. However, it remains unclear how PsbT facilitates the recovery of photodamaged PSII complex.

Possible Function of PsbT in the Repair Process—Close localization of PsbT in the D1/D2 heterodimer suggests that this polypeptide could form a scaffold for the replacement of the photodamaged D1 by a newly synthesized copy. The replacement of D1 could be stimulated by a hydrophobic interaction between PsbT and D1. Alternatively, PsbT might be required for maintaining a structure of the D1-depleted PSII complex. It is expected that the PSII core complex depleted of D1 might accumulate transiently during the damage-repair cycle. If the structure of the D1-depleted PSII complex was modified by the absence of PsbT, the insertion of newly synthesized D1 into the D1-depleted PSII complex and/or a subsequent structural organization of the replaced D1 in the PSII complex would be affected. Accordingly, the absence of PsbT would lead to accumulation of the unstable PSII complexes to a larger extent, which could lead to a decreased steady-state level of PSII complex in strong light.

The repair of the photodamaged PSII also requires assembly and reactivation of PSII cofactors that were lost during D1 replacement. Since D1 binds photosynthetic pigments, plastoquinone, and the oxygen-evolving manganese complex, these cofactors must be assembled into the repaired complex, and reactivated during or after the reinsertion of D1 into a D1-depleted PSII complex. PsbT in the vicinity of the D1/D2 heterodimer might facilitate this process. In order to resolve the recovery steps in which PsbT is specifically involved, it will be necessary to measure partial electron transfer activity of PSII during photoinhibition and recovery processes and estimate contents of redox components such as plastoquinone, plastoquinone, and manganese atom.

Acknowledgments—We thank M. Hippler for critical reading the manuscript and J.-D. Rochaix for providing the transforming plasmid for generating psbT deletion mutants.

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