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Knockdown of the PsbP protein does not prevent assembly of the dimeric PSII core complex but impairs accumulation of photosystem II supercomplexes in tobacco

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ABSTRACT

The PsbP protein is an extrinsic subunit of photosystem II (PSII) specifically found in land plants and green algae. Using PsbP-RNAi tobacco, we have investigated effects of PsbP knockdown on protein supercomplex organization within the thylakoid membranes and photosynthetic properties of PSII. In PsbP-RNAi leaves, PSII dimers binding the extrinsic PsbO protein could be formed, while the light-harvesting complex II (LHCII)–PSII supercomplexes were severely decreased. Furthermore, LHCII and major PSII subunits were significantly dephosphorylated. Electron microscopic analysis showed that thylakoid grana stacking in PsbP-RNAi chloroplast was largely disordered and appeared similar to the stromally-exposed or marginal regions of wild-type thylakoids. Knockdown of PsbP modified both the donor and acceptor sides of PSII; In addition to the lower water-splitting activity, the primary quinone Q_A in PSII was significantly reduced even when the photosystem I reaction center (P700) was noticeably oxidized, and thermoluminescence studies suggested the stabilization of the charged pair, S_2/Q_A^-. These data indicate that assembly and/or maintenance of the functional MnCa cluster is perturbed in absence of PsbP, which impairs accumulation of final active forms of PSII supercomplexes.

1. Introduction

Photosystem II (PSII), the water/plastoquinone (PQ) oxidoreductase, is the supramolecular pigment–protein complex consisting of membrane intrinsic and extrinsic proteins [1]. In addition to the major intrinsic subunits that are involved in pigment and/or cofactor binding for photochemical reaction, PSII has extrinsic subunits protruding into the thylakoid lumen. These play crucial roles in protecting the catalytic manganese cluster [2,3]. PSII catalyzes the water-splitting reaction via light-excited chlorophyll (Chl) molecules with an extremely oxidizing potential, hence plants have developed the precise and regulated system for the coordinated assembly/disassembly of PSII complex to cope with oxidative photodamage.

Among the membrane intrinsic subunits of PSII, the D1 protein is a central functional subunit, of which synthesis/degradation proceeds in a light-dependent manner and turnover rate is higher than that of any other membrane intrinsic subunits [4]. The mechanism to replace photo-damaged D1 effectively and assemble active PSII is called the PSII repair cycle. This cycle has a crucial role in maintaining PSII function in vivo, which includes migration of PSII units containing photoinactivated D1 from grana to stroma regions of thylakoids, partial disassembly of PSII complexes, degradation of D1 by specific proteases, co-translational insertion of the newly synthesized D1 copy in the remaining PSII and final assembly into functional PSII that migrates back to the stacked grana regions [5,6]. During this dynamic cycle, the extrinsic subunits of PSII, also referred to as oxygen-evolving complex (OEC) proteins, are disassembled upon the removal of the damaged D1 and reassembled to protect the manganese cluster [7,8].

It has been known that higher plants and green algae have three OEC proteins, PsbO, PsbP, and PsbQ, while cyanobacteria have a different set of OEC proteins, PsbO, PsbU, PsbV, cyanoP, and cyanoQ [2,3,9,10]. Phylogenic analysis suggested that PsbP and PsbQ in higher plants originated from their cyanobacterial homologs, cyanoP and cyanoQ [11,12]. However, higher plants have PsbP-Like proteins (PPLs) that exhibit higher sequence similarity to cyanoP. *Arabidopsis* has two PPLs

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(PPL1 and PPL2) and both have completely different functions from PsbP; PPL1 is required for the efficient PSII repair under high intensity light illumination, while PPL2 is essential for the stable accumulation of the chloroplastic NAD(P)H dehydrogenase complex [13]. In addition to PPLs, many PsbP homologs as well as PsbQ-Like proteins were also found in the *Arabidopsis* genome [10,13,14]. These facts suggest that after an intensive period of genetic diversification during the evolution of oxygenic photosynthetic organisms, PsbP and PsbQ evolved to control PSII function in chloroplasts of higher plants and green algae.

Molecular function of PsbP and PsbQ is thought to maintain Ca^{2+} and/or Cl^{-} ion in the vicinity of the Mn cluster in PSII [15–17]. The *Chlamydomonas* FUD39 mutant lacking PsbP accumulates a substantial amount of PSII centers, while high concentration of Cl^{-} was necessary to promote oxygen evolution and the light-driven assembly of Mn cluster (termed photoactivation) was significantly slowed [18–20]. In higher plants, in vivo functions of PsbP and PsbQ proteins were firstly characterized in transgenic tobacco in which the levels of PsbP, or PsbQ were severely down-regulated by RNA interference (RNAi) [21]. PsbP-RNAi plants showed retarded growth, pale green leaves, and low PSII activity, whereas PsbQ-RNAi plants were comparable to wild-type plants. As observed in the *Chlamydomonas* mutant, PSII could accumulate without PsbP, whereas the stability of the Mn cluster was largely affected, especially under dark conditions. Later studies using PsbP-RNAi *Arabidopsis* indicated that PsbP was required for the assembly and stability of PSII [22], and PsbQ-RNAi affected the stability of PSII under prolonged dark conditions [23]. Although the phenotypes of PsbP/PsbQ knockout or knockdown are somehow different among the species, it is obvious that PsbP has been developed as a more important component of PSII than PsbQ in higher plants.

Recent studies proposed that the PSII extrinsic proteins might have a more dynamic function in the life cycle of PSII [10,24,25]. In this regard, PsbO is required for the stable accumulation of PSII core complex [26–28], while PsbP is proposed to function in PSII stabilization/assembly [21,22] or to regulate the photoactivation of PSII [29,30]. In the current study, we characterized the biochemical and photosynthetic properties of PSII in PsbP-RNAi tobacco to clarify the physiological role of PsbP for the PSII life cycle. We report that knockdown of PsbP does not prevent PSII core assembly, but affects the stable accumulation of the LHCII–PSII supercomplex; PSII accumulated in the intermediate forms in PsbP-RNAi leaves, while the electron flow was altered to protect PSII from light-induced degradation. The presented data indicate that PsbP regulates the active state of PSII complex in tobacco.

2. Materials and methods

2.1. Plant materials

The transgenic tobacco (*Nicotiana tabacum* cv. Samsun NN), in which *psb*P expression was severely down-regulated by RNAi (PsbP-RNAi: 37bpir No. 14), was previously produced [21]. Another PsbP-RNAi tobacco line having different RNAi trigger sequence (550 bp) was also produced (CDSir No. 9), which showed almost the same phenotype as the previous line [31]. Tobacco plants were grown on agar solidified 0.5× Linsmaier−Skoog (LS) medium supplemented with 1.5% sucrose under continuous light [10 μmol (photons) m^{-2} s^{-1}] at 25 °C. For analytical purposes, tobacco plants (T_2 or T_3 generation) precultivated on LS agar medium were transplanted into soil and grown under continuous light [50 μmol (photons) m^{-2} s^{-1}] at 28 °C. Fully developed leaves (the fourth-sixth leaves from the top) were used in all experiments.

2.2. Preparation of thylakoid membranes

Leaves were homogenized in a blender with the ice-cold buffer 1 (50 mM Hepes–KOH (pH 7.5), 330 mM sorbitol, 1 mM $MgCl_2$, 2 mM EDTA, 0.05% BSA, 5 mM sodium ascorbate). The mixture was filtered and centrifuged (2500 ×*g*, 5 min), and the pellet was resuspended in the buffer 2 (50 mM Hepes–KOH (pH 7.5), 5 mM sorbitol). The solution was centrifuged (2500 ×*g*, 5 min) and the pellet was resuspended in the buffer 3 (50 mM Hepes–KOH (pH 7.5), 100 mM sorbitol, 10 mM $MgCl_2$). The Chl concentration was determined as described in [32]. For phosphothreonine immunoblot analysis, leaves were frozen immediately after excision and 10 mM NaF was included in all the extraction buffer to avoid dephosphorylation.

2.3. SDS-PAGE and immunoblot analysis of proteins

Proteins were separated on 12.5% or 15% SDS-polyacrylamide gels containing 6 M urea. Separated proteins were transferred to a polyvinyliden difluoride (PVDF) membrane using a semidry blotting system (Bio-Rad). Immunoblot detection was performed using an enhanced chemiluminescence system (ECL; Amersham Biosciences).

2.4. Blue native polyacrylamide gel electrophoresis (BN-PAGE)

BN-PAGE was performed as described in [33] with the following modifications. Thylakoids were solubilized in 50 mM Bis–Tris–HCl (pH 7.0), 0.5 M ε-aminocaproic acid, and 10% (v/v) glycerol containing 1% (w/v) *n*-dodecyl-β-D-maltoside and incubated on ice. Samples were centrifuged at 100,000 ×*g* for 10 min at 4 °C. 2.6 μl of 5% Coomassie dye stock solution (5% (w/v) Serva Blue G (Serva, Heidelberg), 50 mM Bis–Tris–HCl (pH 7.0), 0.5 M ε-aminocaproic acid) was added to 80 μl of the supernatant. Proteins were separated on a 4–12% linear gradient polyacrylamide gel.

2.5. Low temperature fluorescence measurements

Low temperature (77 K) fluorescence emission spectra were measured with dark-adapted intact leaves from WT and PsbP-RNAi tobacco. Leaves were soaked in 15% polyethylene glycol 4000 solution and immediately frozen in liquid nitrogen. Fluorescence emission spectra between 650 nm and 800 nm were measured with F-4500 spectrofluorometer (HITACHI, Japan).

2.6. Electron microscopy

Tobacco leaves were fixed with 2.5% glutaraldehyde in a fixing buffer (75 mM sodium phosphate buffer, pH 7.0, containing 2 mM $MgCl_2$), washed several times with fixing buffer, and post-fixed using 1% osmium tetraoxide in the same buffer. The post-fixed segments were dehydrated with ethanol, then propylene oxide, and embedded in epoxy resin. Ultra thin sections (70 to 80 nm thick) were stained with 1% uranyl acetate followed by lead citrate, and examined under a JEM-1220 transmission electron microscope (JEOL, Tokyo, Japan).

2.7. Chl fluorescence and P700 oxido-reduction analysis

Chl fluorescence parameters were measured using a PAM-2000 Chl fluorometer (Walz, Effeltrich, Germany). The minimum Chl fluorescence at an open PSII center in dark-adapted leaves (F_o) was determined using light (655 nm) at an intensity of 0.05–0.1 μmol (photons) $m^{-2}s^{-1}$. The variable fluorescence (F_v) and the minimum Chl fluorescence at an open PSII center in light-adapted leaves (F'_o) was calculated as $F_v = F_m - F_o$ and $F'_o = F_o/\{(F_v/F_m) + (F_o/F'_m)\}$, respectively [34]. A saturation pulse of white light [2500 μmol (photons) m^{-2} s^{-1} for 0.8 s] was applied to determine the maximum Chl fluorescence at closed PSII centers in the dark (F_m) and during actinic light (AL) illumination (F'_m). The steady state of the Chl fluorescence level (F_s) was recorded during AL illumination [26 μmol (photons) m^{-2} s^{-1}]. Photochemical quenching qP was calculated as $(F'_m - F_s)$/

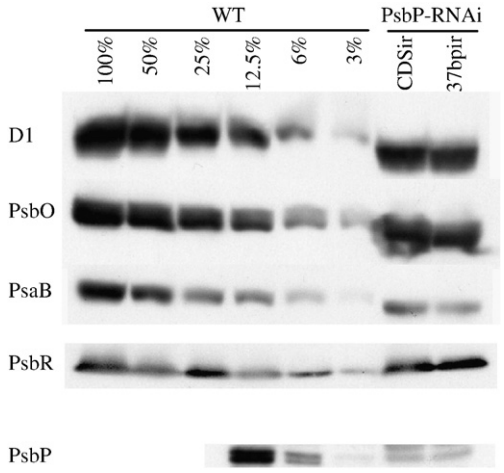

Fig. 1. Immunoblot analysis of thylakoid membrane proteins of wild-type (WT) and PsbP-RNAi thylakoid membranes. Thylakoid proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunodetected with anti-D1, PsbO, PsaB, PsbR and PsbP antibodies, respectively. To detect small amount of PsbP in PsbP-RNAi thylakoids, only diluted sample of WT (<12.5%) were loaded to avoid overexposure. The samples of WT (100%) and PsbP-RNAi contained 5 μg Chl.

($F_m' - F_o'$). The fast induction kinetics (Kautsky curve) was obtained from 1 ms to 2 s after the onset of strong AL.

The change in the absorbance of P700 at 810 nm was measured with a PAM-2000 Chl fluorometer equipped with an emitter–detector unit (ED-P700DW-E) (Walz; [35]). The proportion of oxidized P700 was calculated as $\Delta A/\Delta A_{max}$: ΔA_{max} is the absorption change at 810 nm induced by a saturating far red light (>720 nm) followed by a 50 ms flash from a xenon discharge lamp (XMT-103), and ΔA is that by continuous AL illumination.

2.8. Electron-transfer measurements

The electron transfer through PSII was measured as photoreduction of 2,6-dichlorophenolindophenol (DCIP) at 590 nm with or without exogenous electron donor 2,2′-diphenylcarbonic dihydrazide (DPC) in a photo-diode array spectrophotometer (SCINCO, S-3150, Korea). The assay was performed at a concentration of thylakoids of 10 μg Chl/ml in the buffer containing 20 mM HEPES/NaOH, pH 7.6, 0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, and 5 mM NH_4Cl with 25 μM DCIP as electron acceptor. When necessary, DPC was used to a final concentration of 0.5 mM.

2.9. Thermoluminescence measurements

Thermoluminescence was recorded with an apparatus (Photon System Instruments, Brno). Leaf segments of 9 mm in diameter were incubated on 0.1% Tween20 solution. For Q-band analysis, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of electron transfer from Q_A to Q_B, was added to the solution to a final concentration of 100 μM. The leaf segments were then dark-adapted for 2 min at 25 °C, cooled to −2 °C, illuminated with a short actinic flash (30 μs), and dark-adapted for 90 s. Light emission

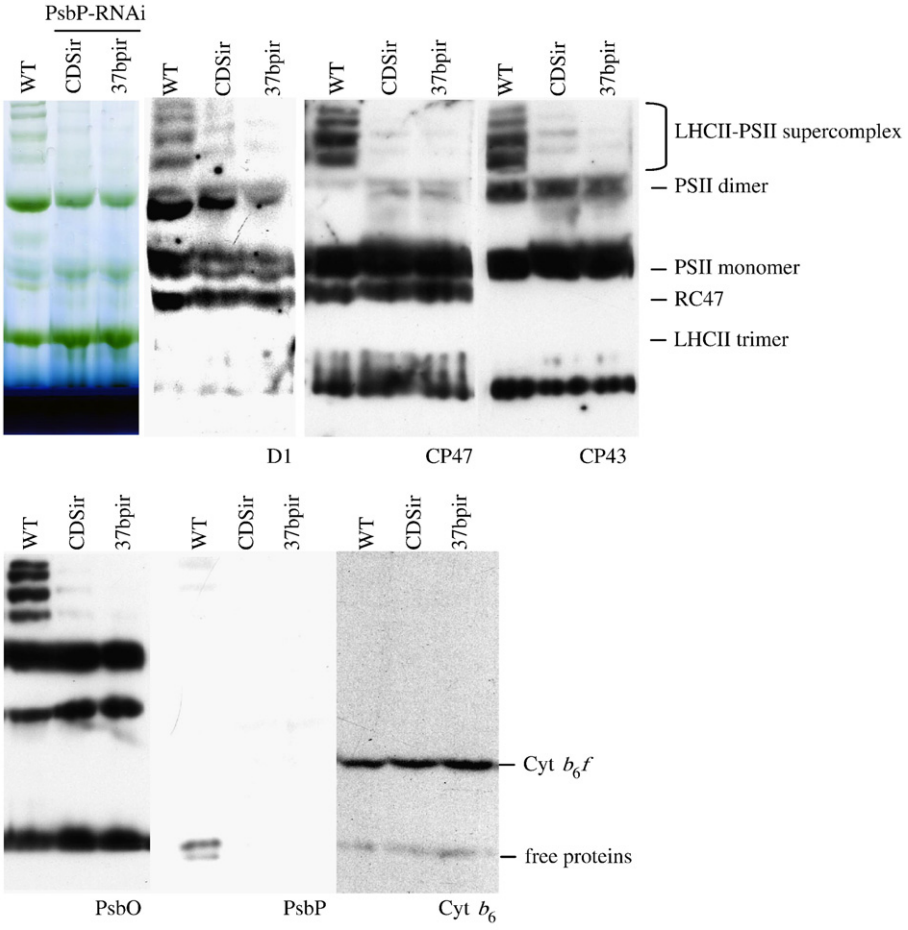

Fig. 2. BN-PAGE of protein complexes on thylakoid membranes. Thylakoids of WT and PsbP-RNAi tobacco leaves were solubilized with 1% *n*-dodecyl-β-D-maltoside and a protein concentration corresponding to 7.5 μg Chl were separated on 4–12% acrylamide linear gradient gels. The gels were used for transferring proteins to PVDF membrane and subsequent immunodetection of D1, CP47, CP43, PsbO, PsbP, and cytochrome (Cyt) b_6 proteins, respectively.

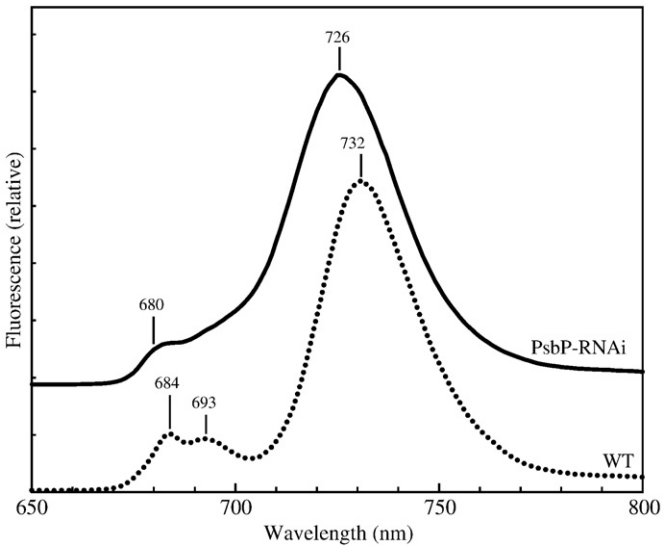

Fig. 3. Low temperature (77 K) fluorescence spectra of WT and PsbP-RNAi leaves. The fluorescence emission was measured from 650 nm and 800 nm with excitation at 435 nm. Three distinct fluorescence peaks, 684 nm (CP43), 693 nm (CP47) and 732 nm (PSI) were shown in WT doted line. In PsbP-RNAi (solid line), additional peaks of 680 nm derived from LHCII was appeared and PSI fluorescence peak was blue-shifted (726 nm).

during sample warming were recorded from −2 °C to 65 °C at 60 °C min^{-1} heating rate.

3. Results

3.1. The LHCII–PSII supercomplexes were decreased in PsbP-RNAi leaves

Conventional SDS-PAGE followed by immunoblotting using specific antibodies showed that accumulation of D1 and PsbO subunits of PSII in PsbP-RNAi tobacco was about 80–100% of that in wild-type (WT) tobacco, and their level were varied by light condition (Fig. 1). The level of PsbR protein, which is reported to be an important link in PSII for stable assembly of PsbP [36], was not changed in PsbP-RNAi leaves. The total level of PsbP, which was immunodetected as doublet bands for four PsbP isoforms in tobacco, was 4–5% of that of WT, indicating that large part of PSII complexes accumulated in PsbP-RNAi leaves lacked PsbP. The level of PsaB of Photosystem I (PSI) was about 30% of WT, which was consistent with the lower Chl *a*/*b* ratio and the level of P700 determined by spectrophotometry previously [21]. In the previous report, we also had observed severe decrease in PsaC of PSI in PsbP-RNAi thylakoids. The reduced level of PSI in PsbP-RNAi leaves would be caused by the low electron supply from PSII to PSI. Because comparable amount of *psa*A and *psa*C mRNA was accumulated in PsbP-RNAi and WT leaves (data not shown), the decrease of PSI in PsbP-RNAi leaves seems to be a consequence of post-transcriptional level regulation.

We then investigated effects of the PsbP knockdown on the formation of protein complexes on thylakoid membranes. In Fig. 2, thylakoid membranes were moderately solubilized with 1% *n*-dodecyl-β-D-maltoside and the protein complexes were separated by BN-PAGE. In PsbP-RNAi thylakoid membranes, the accumulation of LHCII–PSII supercomplexes was remarkably reduced compared to that in WT membranes (Fig. 2). Similar reduction in LHCII–PSII supercomplexes was also observed in the PsbP-RNAi tobacco grown on a sucrose-containing medium (data not shown). Immunoblotting using anti-D1, -CP47, -CP43 and -PsbO antibodies showed that a fraction of PSII dimer, PSII monomer, and PSII monomer lacking CP43, called RC47 complex, were accumulated in PsbP-RNAi leaves. We could not detect PsbP in any protein complexes and only detected as lower bands in WT membranes. This is because PsbP is known to dissociate easily from PSII complex during solublization by *n*-dodecyl-β-D-maltoside and subsequent BN-PAGE [37,38].

To confirm functional dissociation of LHCII from PSII reaction center, 77 K fluorescence spectra of intact leaves without invasive treatment were measured (Fig. 3). In PsbP-RNAi leaves, the extra peak around 680 nm characteristic of free LHCII was appeared, while the peaks at 684 nm (originating from Chl *a* of CP43) and 693 nm (CP47), which were observed distinctly in WT leaves, were obscured. In addition, the emission peak originating from antenna pigments of PSI was significantly blue-shifted from 732 nm in WT to 726 nm in PsbP-RNAi leaves, which might be caused by a change around PSI complex (Fig. 1) or a marked decrease of LHCI [21]. The data indicate that a decrease in energy transfer from LHCII to PSII core and support the observation in BN-PAGE analysis (Fig. 2) that shows the dissociation of the LHCII antenna from the PSII dimer in PsbP-RNAi leaves. Similar observation was reported on the ΔPsbJ mutant that concomitantly lacks PsbP and PsbQ [39]. Therefore, we conclude that PSII dimer with PsbO was formed even in PsbP-RNAi leaves, whereas accumulation of PSII supercomplexes is vastly reduced upon depletion of PsbP.

3.2. Major PSII subunits and LHCII are dephosphorylated in PsbP-RNAi leaves

Phosphorylation levels of proteins on thylakoid membranes were analyzed by immunoblotting using anti-phosphothreonine antiserum. Phosphorylation of LHCII is known to be associated with the relocation of LHCII between PSII to PSI, and thus is involved in redistribution of excitation energy between PSII and PSI (state transition). The physiological function of the phosphorylation of PSII core proteins has been unclear, but recent study suggests that it is needed for PSII disassembly and the lack of phosphorylation leads to accumulation of damaged PSII center [40]. As shown in Fig. 4B, LHCII was remarkably dephosphorylated in PsbP-RNAi leaves compared to that in WT leaves

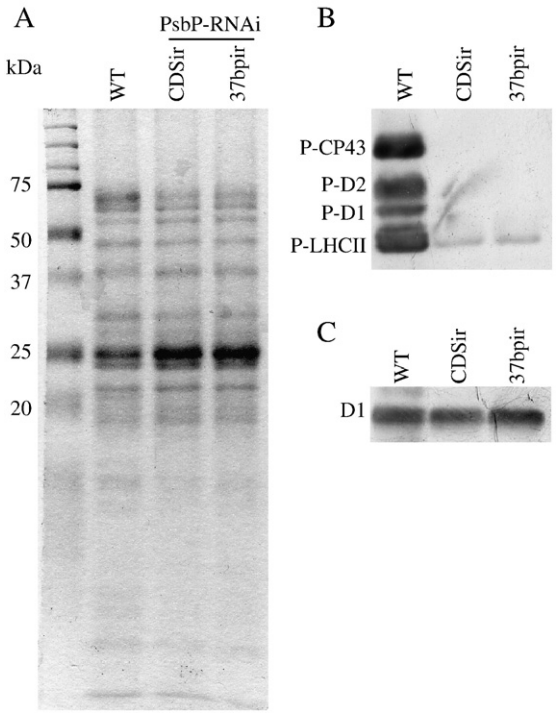

Fig. 4. Immunoblot analysis of phosphoproteins and D1 protein on thylakoid membranes in WT and PsbP-RNAi leaves. (A) Proteins corresponding to 5 μg Chl were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. (B, C) Immunodetection using anti-phosphothreonine antibody and anti-D1 protein, respectively. Proteins corresponding to 2.5 μg Chl were loaded in each lane.

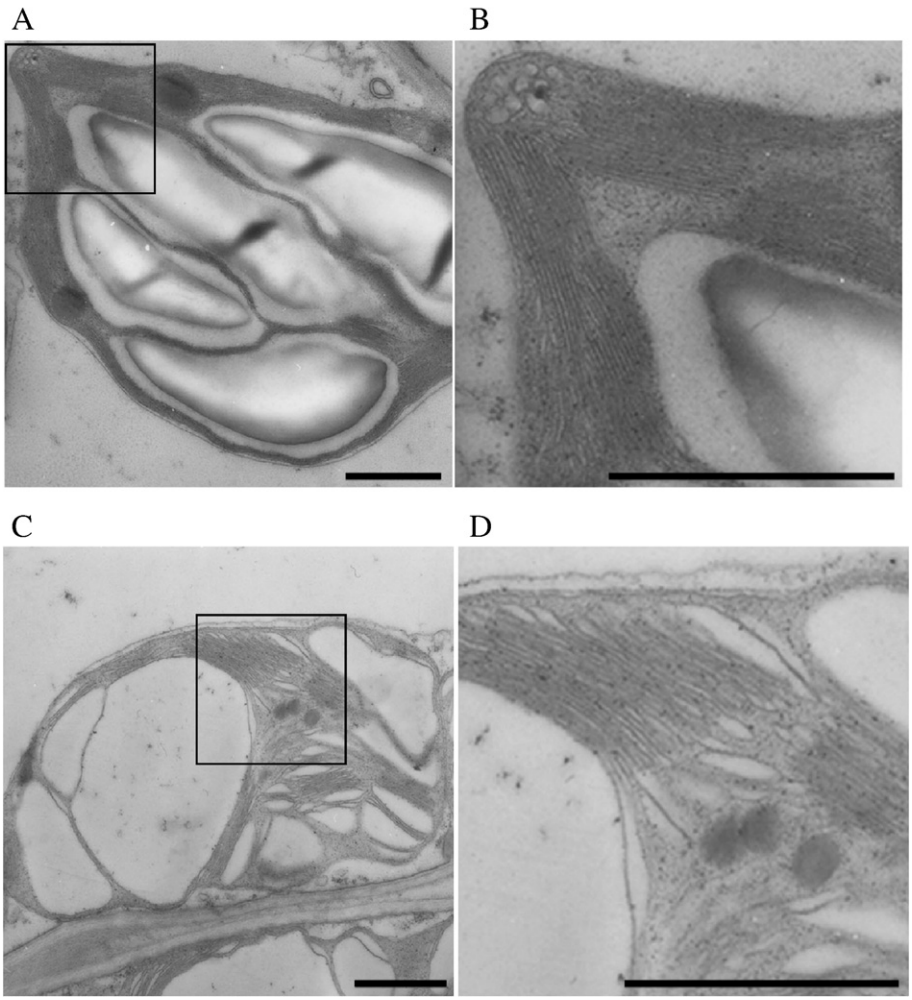

Fig. 5. Electron microscopic photographs of WT (A and B) and PsbP-RNAi (37bpir No. 14; C and D) tobacco chloroplasts. Mature leaves of plants grown in moderate light conditions [~50 μmol (photon) m^{-2} s^{-1}, continuous light] were used for the observation. Areas surrounded by black boxes in A and C are enlarged in B and D, respectively. Bars = 1 μm.

under continuous light condition [50 μmol (photons) m^{-2} s^{-1}]. PSII core subunits (D1, D2, and CP43) were also remarkably dephosphorylated in PsbP-RNAi leaves, while similar amounts of thylakoid proteins and D1 were accumulated in WT and PsbP-RNAi thylakoids (Fig. 4A and C). Phosphorylation of LHCII is known to be controlled by the redox state of the PQ pool and also by ferredoxin–thioredoxin system in stroma [41]. Since the activity of PSII was severely impaired in PSII-RNAi tobacco [21], it is reasonable to assume that the PQ pool in PsbP-RNAi thylakoid would be in oxidized state. On the other hand, regulation of phosphorylation of PSII core subunits is less understood. Defect in phosphorylation of PSII core subunits is similarly observed in higher plants having mutations in genes encoding PsbI and PsbTc [37,42]. Both mutants also lack stable LHCII–PSII supercomplexes, and it was assumed that phosphorylation of PSII core proteins were blocked by structural changes of PSII itself, which is also likely in PsbP-RNAi leaves.

3.3. The grana stacks were largely disordered in PsbP-RNAi chloroplast

In order to examine effect of PsbP-RNAi on the ultrastructure of thylakoid membranes, the structural organization of chloroplasts was analyzed by transmission electron microscopy. The thylakoid membrane system in chloroplasts has functionally differentiated structures; decomposition and reassembly of the photo-damaged PSII as well as its *de novo* synthesis occur in stromally-exposed regions, and active LHCII–PSII supercomplexes accumulate in the stacked grana (for review, [43]). The biochemical properties of dephosphorylated and LHCII-detached PSII in PsbP-RNAi leaves resemble the intermediates of PSII repair cycle that is thought to be present in the stromally-exposed or marginal region of the thylakoids. The electron microscopic studies shown in Fig. 5 revealed that granal stacking was apparently destablished and no starch grains were observed in the chloroplast of PsbP-RNAi leaves, while large starch granules and an ordered structure of grana stacks were observed in WT. Such changes were not observed in PsbQ-RNAi leaves (data not shown). A recent study has suggested that from analyzing the height of grana membrane stacks, lumenal protrusions of adjacent photosystem II complexes in opposing membranes are likely to be displaced relative to each other [44]. This suggests that PsbP and PsbQ are not directly involved in grana stacking. On the other hand, it was reported that the removal of PsbP and PsbQ resulted in a considerable shift in the position of the minor antenna protein CP29, and slight displacement of LHCII associated with PSII [45]. Knockdown of PsbQ does not affect accumulation of LHCII–PSII supercomplexes (Suppl. Fig. 1). Therefore, it is reasonable to assume that PsbP is required for stable LHCII association with the PSII dimer, and concomitant decrease in the amount of LHCII–PSII supercomplex might prevent the formation of stable grana stacking in PsbP-RNAi leaves. As a result, domain sectioning of thylakoid membranes between the grana and stromally-exposed region could become obscure in PsbP-RNAi leaves, and this obscure sectioning would enable the enhanced accumulation of intermediate forms of PSII.

Table 1
Electron transport rate of WT and PsbP-RNAi (37bpir No. 14) thylakoid estimated by DCIP reduction, and the redox state of Q_A and P700 in WT and PsbP-RNAi leaves.

	DCIP − DPC[a]	DCIP + DPC[a]	qP[b]	$P700^+/P700_{total}$[c]
WT	154.40 ± 32.25 (100%)	200.68 ± 27.76 (100%)	0.953 ± 0.003	0.255 ± 0.045
PsbP-RNAi	17.55 ± 16.58 (11.3%)	96.10 ± 10.33 (47.9%)	0.585 ± 0.039	0.818 ± 0.099

Data presented are means ± SD ($n = 3$).
[a] μmol DCIP reduced mg Chl^{-1} h^{-1}. Photoreduction of DCIP at 590 nm in the absence or presence of DPC was measured. Thylakoid membranes corresponding to 10 μg Chl ml^{-1} were used.
[b] The proportion of oxidized Q_A (qP) in leaves under 26 μmol (photons) m^{-2} s^{-1} actinic light was estimated from Chl fluorescence measurement.
[c] The proportion of oxidized P700 in leaves under 26 μmol (photons) m^{-2} s^{-1} actinic light was estimated from the absorbance change at 810 nm.

3.4. Both donor and acceptor sides of PSII were modified in PsbP-RNAi leaves

The functional properties of PSII centers were investigated in PsbP-RNAi leaves. First, photosynthetic electron transport activity of PSII was measured in isolated thylakoid membranes using DCIP as an artificial electron acceptor. To compare the activities of PSII centers capable of water splitting in WT and PsbP-RNAi thylakoid membranes, DCIP reduction rate was measured in the absence of the artificial electron donor DPC. Thereafter, to compare the activities of PSII centers capable of electron transfer, DCIP reduction rate was measured in the presence of DPC. A small increase in the reduction rate of DCIP with DPC in WT thylakoids was observed. This was due to electron transfer from PSII centers without a functional Mn cluster in addition to the active centers (Table 1). In PsbP-RNAi thylakoids, DCIP reduction activity was very low in the absence of DPC and significantly activated by DPC, suggesting that a large part of PSII centers were active in electron transport, but could not oxidize water effectively. It should be noted that the population of the active Mn cluster in actual PsbP-RNAi leaves would be higher than that estimated in isolated thylakoids, since the Mn cluster in PsbP-RNAi leaves was very unstable and could be readily dissociated during the isolation procedure [21]. At the same time, a certain fraction of PSII centers could not transport electron from DPC to DCIP effectively in PsbP-RNAi thylakoids.

To investigate the photosynthetic linear electron flow in PsbP-RNAi leaves, the redox states of Q_A in PSII and P700 in PSI under moderate light illumination were determined. We previously reported that not only the water-splitting activity, but also subsequent electron transfer was impaired in intact PsbP-RNAi leaves; higher steady-state Chl fluorescence (F_s) level under actinic light and the slow fluorescence quenching after the saturation pulse in the dark indicated that re-oxidation of the primary quinone Q_A was slowed in PsbP-RNAi leaves [21,22]. The slow re-oxidation of Q_A^- in PsbP-RNAi leaves might be caused by the reduced amount of PSI as shown in Fig. 1, while low phosphorylation level of LHCII in PsbP-RNAi leaves suggests the oxidized state of the PQ pool. To estimate the redox state of Q_A, we evaluated the parameter qP, which is inversely related to the reduction status of Q_A. To analyze the redox state of P700, the absorption change of 810 nm was measured and the proportion of oxidized P700 was calculated. As shown in Table 1, qP in PsbP-RNAi leaves was lower than that in WT leaves, while the proportion of oxidized P700 in PsbP-RNAi leaves was higher than that in WT. Similar results were observed using a range of moderate light intensity (data not shown). These results suggest that suppression of linear electron flow was not caused by the reduced amount of PSI in PsbP-RNAi leaves, and that the slow re-oxidation of Q_A^- in PsbP-RNAi leaves would be due to a change inside PSII.

We then analyzed the fast induction kinetics of Chl fluorescence during the onset of the actinic light in order to analyze the reduction process of Q_A within PSII (Fig. 6). The Chl fluorescence rises from a minimum F_O level to a peak F_P level through a local minimum F_I level

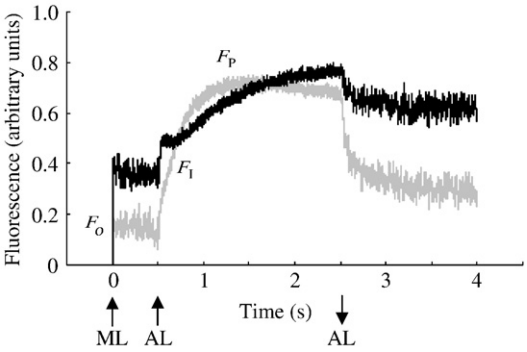

Fig. 6. Induction kinetics of Chl fluorescence during dark to light transition. The locations of the F_O, F_I and F_P of WT are indicated. Black line, PsbP-RNAi (37bpir No. 14); Gray line, WT. Upward or downward arrows indicates onset or offset of light illumination. AL, actinic light; ML, measuring light.

(Kautsky curve; [46]). In WT leaves, the fluorescence reached to the F_P level within 1 s after the onset of actinic light. However, F_O level in PsbP-RNAi leaves was higher than that in WT leaves, indicating that PSII antenna and core is disconnected in accordance with Figs. 2 and 3. In addition, a fluorescence quenching was observed after the F_I level in PsbP-RNAi leaves, and the time to reach the F_P level was significantly longer (~2 s). This result indicates that after the accumulation of Q_A^- to the F_I level, its further accumulation was slowed in PsbP-RNAi leaves. This would be due to poor excitation energy transfer from antenna to PSII reaction center and poor electron supply from

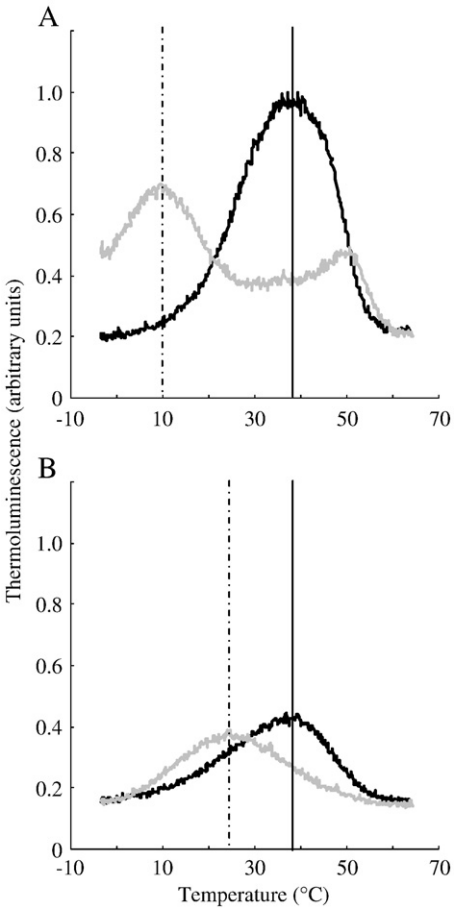

Fig. 7. Thermoluminescence glow curves of $S_2Q_B^-$ (black) and $S_2Q_A^-$ (gray) charge recombination in (A) WT and (B) PsbP-RNAi (37bpir No. 14) leaves. Vertical broken lines and solid lines represent temperatures at which Q-bands derived from $S_2Q_A^-$ and B-bands derived from $S_2Q_B^-$ charge recombination were observed, respectively.

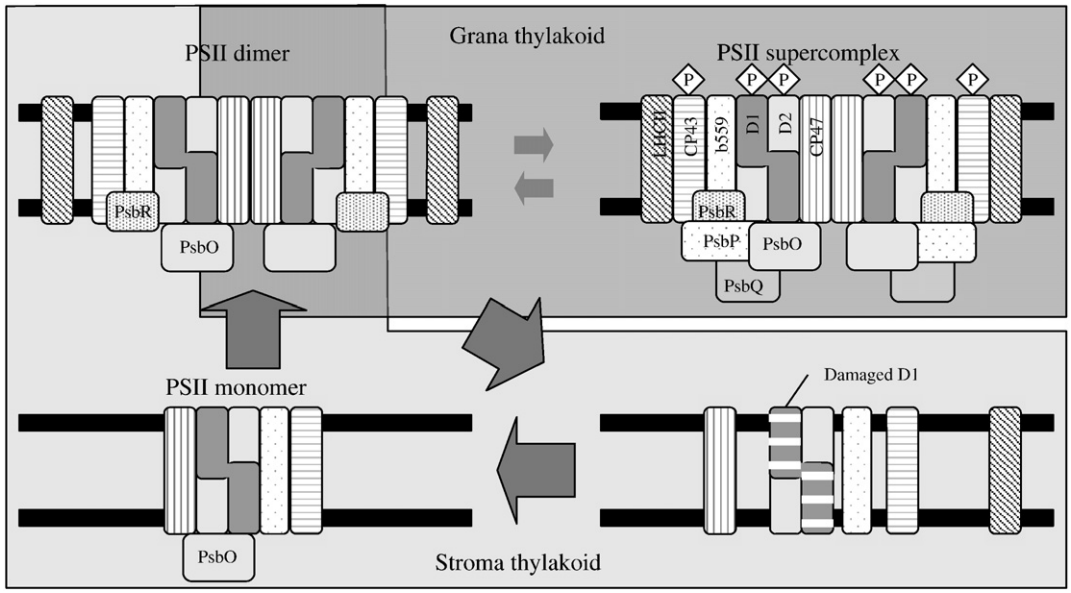

Fig. 8. A schematic representation of the PSII complexes in PsbP-RNAi tobacco leaves. These models are designed to aid the reader in conceptualizing the PSII complexes observed in the PsbP-RNAi tobacco, but by no means show the exact location of each PSII subunit. P: phosphate. See "Discussion" for details.

perturbed oxygen-evolving clusters in PsbP-RNAi leaves, which would promote direct recombination reaction between Q_A^- and positive charges on the donor side, such as $TyrZ^+$ or $P680^+$ [47]. Finally, higher fluorescence level after switching off the actinic light in PsbP-RNAi leaves would be caused by the slower re-oxidation of Q_A^- [21,22] and/or further disconnection of LHCII from PSII core by AL illumination.

3.5. The $S_2Q_A^-$ charge pair within PSII was stabilized in PsbP-RNAi leaves

To investigate the stability of charge pairs within PSII, thermoluminescence measurements were conducted on WT and PsbP-RNAi leaves. Thermoluminescence originates from a PSII reaction center that is re-excited by a charge recombination with an increase in the temperature of samples, where light-induced charge pairs in PSII are freeze-trapped [48]. A B-band arises from a recombination of S_2 state of the Mn cluster with Q_B^- and a Q-band arises from a recombination of the S_2 with the Q_A^-. The temperatures at which the bands are observed indicate the relative stability of the charge pairs. The leaf discs were incubated under dim light and dark adapted for 2 min before measurements. This short dark adaptation is to prevent the dark reduction of the Mn cluster in PsbP-RNAi leaves [21]. In both WT and PsbP-RNAi leaves, the B-bands obtained in the absence of DCMU were observed around 37 °C, indicating that the $S_2Q_B^-$ charge pair stability was not affected in PsbP-RNAi leaves as observed previously (Fig. 7; [21]). On the other hand, the temperature at which Q-bands were obtained in the presence of DCMU was around 10 °C in WT leaves, whereas it was around 24 °C in PsbP-RNAi leaves. This significant up-shift of the temperature generating Q-band in PsbP-RNAi leaves was also observed when phenolic herbicide dinoseb was used instead of DCMU (data not shown). The up-shift of the Q-band indicates that the $S_2Q_A^-$ charge pair was significantly stabilized in PsbP-RNAi leaves. This is consistent with the slow oxidation of Q_A^- observed in PsbP-RNAi tobacco and *Arabidopsis* [21,22, and Fig. 6 in this study] At the same time, lower intensities of the B-band and Q-band in PsbP-RNAi leaves indicate that the charge recombination of a large fraction of the PSII occurs via alternative routes that may not involve the primary charge-separated state and related photon emission.

4. Discussion

It has been hypothesized that the OEC proteins participate in regulation of structural integrity of the PSII supercomplex and grana stacking in higher plants [25]. However, the precise effects of knockout or knockdown of the OEC proteins on the PSII assembly and repair status, as well as the thylakoid ultrastructure, have not been characterized well. In this study, we showed that PsbP-RNAi in tobacco caused the marked decrease in the LHCII–PSII supercomplex and the accumulation of PSII subcomplexes in which both donor and acceptor side reactions were modified as indicated by the lower water-splitting activity and suppressed liner electron flow (Table 1). Furthermore, we showed that grana stacks in thylakoids were significantly disordered in PsbP-RNAi chloroplast. These results suggest that PsbP functions in very crucial steps of PSII life cycle in tobacco.

The present results indicate that PsbP is one of the important factors for the water-splitting activity of PSII in higher plants. In a *Chlamydomonas* PsbP mutant, PSII subunits accumulate normally, while apparent photoactivation is slowed and are competing with photoinhibition [49]. Similarly, photoactivation of the Mn cluster can occur in PsbP-RNAi tobacco, while photoactivation was observed under the dim light, and the PSII activity was hypersensitive to light [21]. This would be caused by instability of the Mn cluster without PsbP, but it is also possible that photoactivation might occur more efficiently in the presence of PsbP as recent studies suggest that PsbP binds Mn and facilitates Mn cluster assembly and restoration of oxygen evolution [29,30]. To prove the latter possibility, it will be important to determine the metal binding site in PsbP that was not seen in the current PsbP crystal structure [50].

Differential silencing of four PsbP isoforms in tobacco revealed that PSII could be accumulated without PsbP, but its activity was directly correlated with the amount of PsbP [51]. This result indicates that PsbP indeed regulates the activity of PSII, but not PSII core assembly. On the contrary, it was reported that PsbP is required for PSII accumulation, especially D2 and CP47 subunits in *Arabidopsis* [22]. This phenotypic difference would be caused by difference in the amount of residual PsbP. Presumably, PsbP has dual functions to regulate assembly and activity of PSII in tobacco; Only 5% of PsbP allows the accumulation of PSII core complexes, while 100% expression of PsbP is required for the optimum PSII activity. Recent study reported that in the extreme halophyte *Salicornia veneta*, the amount of PsbP is sub-stoichiometric with other PSII subunits and likely to function as an assembly factor for PSII [52]. This suggests that physiological roles of PsbP would be different among the species living in different ionic environments.

The photosynthetic properties of PSII in PsbP-RNAi tobacco were observed to be similar to those seen in vitro in spinach PSII-enriched membranes depleted in Ca^{2+} and/or Mn; the depletion of Ca^{2+} modifies the electron transfer on both the donor and acceptor sides of PSII [47]; and the potential of Q_A/Q_A^- was up-shifted by 150 mV when Ca^{2+} or both Ca^{2+} and Mn were lost from PSII [53]. The up-shift of the redox potential of the Q_A/Q_A^- redox couple in the absence of Ca^{2+} would stabilize $S_2Q_A^-$ charge pair. Ono et al. studied the effect of PsbP removal on the Q-band in membranes and showed that PsbP needs to be present to see an up-shifted Q-band under Ca-depleted PSII membranes [54]. Thereafter, it was reported that an up-shift Q-band under Ca-depleted PSII membranes was observed without PsbP in the presence of cryoprotectants [55]. Therefore, it is probable that such solutes may present in intact leaves.

Coupled donor and acceptor side inhibition of PSII was also observed in the FUD39 mutant of *Chlamydomonas* lacking PsbP [20], suggesting a similar change within PSII might be expected to occur eventually in both *Chlamydomonas* and tobacco mutants. These changes would protect PSII from damage caused by light by changing the dominant charge recombination pathway to one which does not involve formation of the $P680^+Ph^-$ radical pair, the P680 triplet, and singlet oxygen [56,57]. The lower thermoluminescence emission of PsbP-RNAi leaves compared with that of WT (Fig. 7) supports this view. Quantitative studies on electron flow using spinach chloroplast suggest that such an alternative electron flow in PSII occurs when pH in thylakoid lumen is lowered and Q_A is in reduced form [58]. Acidification of the thylakoid lumen facilitates the release of Ca^{2+} from PSII [59,60], presumably via the partial dissociation of PsbP. The absence of PsbP would cause a shortage of Ca^{2+} in PSII without a significant acidification of thylakoid lumen, and thus only accumulation of Q_A^- would induce the alternative electron flow within PSII in PsbP-RNAi leaves. This altered electron flow protects PSII from photoinhibition [61] and would allow the accumulation of PSII intermediates in PsbP-RNAi leaves.

From these data along, with those from previous studies, we illustrated a model for PSII complexes in PsbP-RNAi tobacco (Fig. 8). Biochemical characterization of PSII in PsbP-RNAi leaves has showed that PSII, without PsbP, can accumulate as a dimer associated with PsbO, even though it is dephosphorylated and detached from LHCII. The LHCII–PSII supercomplexes seems to be formed in PsbP-RNAi mutant but are fairly decreased, suggesting that the rate of assembly or the stability of LHCII–PSII supercomplex would be impaired in PsbP-RNAi tobacco. Some PSII intermediates without PsbP are protected by the direct recombination between the PSII acceptor and donor sides as described above, while others are irreversibly damaged and enter the repair cycle. Although our BN-PAGE could not detect PsbP in any PSII assemblies, it was proposed that initial binding of PsbO to the thylakoid membrane occurs primarily in the stromally-exposed membranes, and then migrates to granal region followed by PsbP assembly in the granal thylakoid membranes [8]. Consistent with this model, proteomic studies of polysome fraction on *Arabidopsis* thylakoid membrane identified PsbO but not PsbP [62]. In future studies, dynamics of OEC proteins assembly to PSII core complex should be analyzed more precisely.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.03.004.

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