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The N-terminal domain of Lhcb proteins is critical for recognition of the LHCII kinase

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ABSTRACT

The light-harvesting chlorophyll (Chl) *a*/*b* complex of photosystem (PS) II (LHCII) plays important roles in the distribution of the excitation energy between the two PSs in the thylakoid membrane during state transitions. In this process, LHCII, homo- or heterotrimers composed of Lhcb1–3, migrate between PSII and PSI depending on the phosphorylation status of Lhcb1 and Lhcb2. We have studied the mechanisms of the substrate recognition of a thylakoid threonine kinase using reconstituted site-directed trimeric Lhcb protein–pigment complex mutants. Mutants lacking the positively charged residues R/K upstream of phosphorylation site (Thr) in the N-terminal domain of Lhcb1 were no longer phosphorylated. Besides, the length of the peptide upstream of the phosphorylated site (Thr) is also crucial for Lhcb phosphorylation *in vitro*. Furthermore, the two N-terminal residues of Lhcb appear to play a key role in the phosphorylation kinetics because Lhcb with N-terminal RR was phosphorylated much faster than with RK. Therefore, we conclude that the substrate recognition of the LHCII kinase is determined to a large extent by the N-terminal sequence of the Lhcb proteins. The study provides new insights into the interactions of the Lhcb proteins with the LHCII kinase.

1. Introduction

Photosynthetic organisms require various photoacclimation mechanisms to cope with changing growth environments. In order to adapt to the constantly changing light conditions, these organisms have developed mechanisms that protect the photosynthetic apparatus from photo-oxidative damage. Regulating the distribution of excitation energy between photosystem (PS)I and PSII *via* state transitions is one of the mechanisms for adapting to the fluctuating light environment [1,2]. State transitions operate through the reversible migration of the major light-harvesting complexes of PSII (LHCII) between the two PSs through phosphorylation/dephosphorylation of LHCII. The protein kinases for LHCII phosphorylation have been identified in a genetic screen by taking advantage of the large fluorescence changes which occur during state transitions. They are STN7 in *Arabidopsis*[3] and Stt7 in *Chlamydomonas reinhardtii*[4].The activities of the kinase(s) are regulated by the redox state of the plastoquinone pool [5] and triggered by the docking of plastoquinol to the Qo site of the cytochrome(Cyt)b_6f complex [6,7]. Moreover, the Stt7 kinase was found to be in close interaction with the Rieske protein of Cyt b_6f[8]. In higher plants, only 20–25% of LHCII participate in the lateral migration between the two photosystems [3,9], whereas up to 80% of LHCII is mobile in green algae [10]. However, the assumed link between LHCII phosphorylation and migration has been challenged recently [11–13] because some phosphorylated LHCII remains associated with PSII supercomplexes and LHCII serve as an antenna of both PSs under most natural light conditions.

LHCII, the most abundant Chl *a*/*b* complex, accounting for roughly one third of the total membrane proteins in thylakoid membranes, plays an important role in state transitions. Under state 1 conditions, namely when the excitation pressure on PSII is low, LHCII associates mainly to PSII to form the PSII–LHCII supercomplex, while it migrates to PSI to form the PSI–LHCI–LHCII supercomplex under state 2 conditions, when the excitation pressure on PSII increases [14]. The LHCII complexes are homo- or heterotrimers composed of Lhcb1, Lhcb2 and Lhcb3 in higher plants. Normally, Lhcb1 is the most abundant isoform of LHCII. The ratio of Lhcb1/Lhcb2/Lhcb3 is about 8:3:1 in *Arabidopsis*[15]. Mature Lhcb1 and Lhcb2 have a phosphorylation site in their N-terminal domain that undergoes phosphorylation/dephosphorylation during state transitions. In contrast, Lhcb3 cannot be phosphorylated, because it lacks the N-terminal 14 residues that include the phosphorylation sites. Lhcb2 protein shows faster phosphorylation kinetics during state transitions compared with Lhcb1 [16,17]. Lhcb proteins are encoded by a multigene family [18]. In *Arabidopsis* there are five genes for Lhcb1, four for Lhcb2, and only one copy for Lhcb3 [15,18]. Some of these genes lie in close proximity on the chromosomes, and therefore, it is very difficult to generate Lhcb1 or Lhcb2 knockout

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plants [19,20]. This is a serious limitation for elucidating the individual roles of Lhcb1 and Lhcb2 *in vivo*. However significant progress has been achieved recently by Pietrzykowska *et al.*[21] who generated artificial microRNA *Arabidopsis* lines deficient in either Lhcb1 or Lhcb2. This study showed that in spite of their high sequence identity these two proteins have distinct complementary roles.

In order to understand the mechanisms regulating the phosphorylation of the various thylakoid proteins, it is important to determine how different kinases interact with their substrates, a process that is still largely unknown. Although it has been proposed that LHCII could be phosphorylated at one or more sites based on a phosphorylation assay with a series of truncated recombinant proteins in reconstituted LHCII complexes [22,23], the question still remains how the kinases recognize and interact with their Lhcb substrates and which particular structural features of Lhcb2 contribute to its rapid phosphorylation kinetics.

The interaction between the mature Lhcb1 proteins and their kinases has long been a central topic with regard to energy balance in photosynthetic membranes. Sequence alignments indicate that Lhcbs and the activation loop in the Stt7/STN7 kinase are highly conserved in different species. Conserved positively charged residues (R/K) around the phosphorylation site (Thr) in Lhcbs, in parallel with five negatively charged residues in the activation loop of the Stt7/STN7 chloroplast Ser–Thr protein kinase, are present in all photosynthetic organisms examined. In this work, a mutational study was conducted to assess the importance of the N-terminal sequence of Lhcbs for the phosphorylation activity of a thylakoid protein extract containing STN7. Our results indicate that specific changes in the N-terminal domain of Lhcb1 or Lhcb2 strongly affect the phosphorylation of these proteins. This work may provide a basis for further studies of the interactions between Lhcbs and the LHCII kinase, and give new insights into the physiological significance of the different phosphorylation/dephosphorylation kinetics of the different antenna protein subunits.

2. Materials and methods

2.1. Preparation of different Lhcbs and their mutant proteins

The plasmids for Lhcb1 and Lhcb2 overexpression described in [24] were used for Lhcb1 or Lhcb2 apoprotein. The apoproteins were expressed and purified as described previously [24]. Then, the apoproteins were reconstituted with thylakoid pigments with the standard protocol for LHCII folding published previously [25]. The reconstituted pigment protein complexes were loaded onto a sucrose density gradient (10% to 40% sucrose, 0.1% n-dodecyl-β-D-maltoside (DDM), and 5 mM phosphate buffer (pH 8)), and centrifuged at 230,000 × *g* at 4 °C for 16 h (SW-40 rotor, Beckman, Palo Alto, CA). After ultracentrifugation, the bands corresponding to the LHCII trimer were harvested for the phosphorylation assay.

Site-directed mutagenesis to Lhcb1 and Lhcb2 were carried out with a MutanBEST site-directed mutagenesis Kit (Takara Inc., Japan). The mutagenesis primers designed for mutagenesis are listed in Supplementary Table S1. The mutant DNA sequences were verified by DNA sequencing. The apoproteins of the different Lhcb mutants were overexpressed and isolated and reconstituted with thylakoid pigments as described.

2.2. Plant material and short-term light treatments of plants

Pea (*Pisum sativum L.*) and *Arabidopsis* (*Arabidopsis thaliana L.*) were grown under a 14 h photoperiod under an irradiance of 100 μmol photons $m^{-2}s^{-1}$. The temperature and relative humidity were set at 23/19 °C (day/night) and 70%, respectively. Two week old plants were exposed for 3 h to light favoring the excitation of PSI (PSI light) to ensure maximal dephosphorylation of thylakoid proteins, and subsequently subjected to low light illumination (LL:30 μmol photons $m^{-2}s^{-1}$).

The WT *C.reinhardtii* 137c strain was grown as described in [8] in TAP medium, to a density of OD750 = 1.0.

2.3. Thylakoid membrane preparation from different species

Cells of *C. reinhardtii* 137c were harvested by centrifugation (6000 × *g*, 10 min), and the pellet was resuspended in an isolation buffer (0.3 M sucrose, 25 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$). Cells were broken by vortexing in the presence of glass beads (Sigma, Inc., USA) in the isolation buffer. Unbroken cells were removed by centrifugation (1000 × *g*, 1 min), and the membranes were collected from the supernatant by further centrifugation for 10 min. The membranes in the pellet were collected and resuspended in a 25 mM Tricine–NaOH (pH 8.0)/100 mM Sorbitol/20 mM NaCl/5 mM $MgCl_2$ buffer.

Leaves from *Pea* or *Arabidopsis* were harvested and homogenized by vortexing in a buffer containing 0.3 M sucrose/25 mM Hepes-KOH (pH 7.5)/1 mM $MgCl_2$. The homogenate was subjected to centrifugation (1000 × *g*, 1 min). The resulting supernatant was centrifuged again (20,000 × *g*, 10 min), and the membranes were recovered from the pellet which was resuspended in a buffer (25 mM Tricine–NaOH (pH 8.0)/100 mM Sorbitol/20 mM NaCl/5 mM $MgCl_2$).

2.4. Preparation of crude thylakoid kinase extract

The crude thylakoid kinase extract was prepared from thylakoids of spinach purchased from farmers. The leaves were solubilized in n-octyl-β, D-glucopyranoside and N-cholate, and the crude kinase preparation containing Cyt b_6f, was obtained by ammonium sulfate precipitation following the protocol described previously [26]. The kinase composition of the extract was determined based on immunoblotting or LC-MS/MS analysis. The crude kinase preparation corresponding to 0.1 μg Cyt *f* was separated by Tris-SDS-PAGE, followed either by immunoblot analysis using specific antibodies against STN7 or STN8 (Abcam, Inc., USA), or by LC-MS/MS analysis.

2.5. LC-MS/MS analysis and database search

Gel slices from 25 kDa to 100 kDa in the Tris-SDS-PAGE of the crude thylakoid extract were cut and subjected to trypsin digestion (Trypsin Gold, mass spectrometry grade, Promega) according to [27]. Peptide separation was performed on an Eksigent Ultra 2D plus (AB SCIEX, Inc., USA). Samples were desalted on a 100 μm × 20 mm trap column and eluted on an analytical 75 μm × 150 mm column. Both trap column and analytical column were filled with MAGIC C18AQ, 5 μm, 200 Å phase (Michrom Bioresources, Inc., USA). Peptides were separated by a gradient formed by 0.1% formic acid (mobile phase A) and 100% acetonitrile, 0.1% formic acid (mobile phase B), from 5 to 30% of mobile phase B in 15 min at a flow rate of 300 nl per minute. The MS analysis was performed on a Triple TOF 5600 + system (AB SCIEX, Inc., USA) in Information Dependent Mode. MS spectra were acquired across the mass range of 350–1500 *m/z* in high resolution mode (>30,000) using 250 ms accumulation time per spectrum. A maximum of 40 precursors per cycle were chosen for fragmentation from each MS spectrum with 100 ms minimum accumulation time for each precursor and dynamic exclusion for 20 s. Tandem mass spectra were recorded in high sensitivity mode (resolution >15,000) with rolling collision energy on.

Peptide identification was conducted in the Protein Pilot 4.5 software (AB SCIEX, Inc., USA) using the Paragon database search algorithm and the integrated false discovery rate (FDR) analysis function. The software used only unique peptide sequences as evidence for protein identification. The data were searched against NCBI nr (ver.20150104) containing 54,183,042 proteins entries. For FDR determination, data were searched against concatenated databases by *in silico* on-the-fly reversal for decoy sequences automatically by the software. Only proteins at 1% critical FDR were used for further analysis.

2.6. In vitro phosphorylation assay

A: Phosphorylation with crude kinase preparation: The phosphorylation reaction was performed in a reaction buffer (50 mM Tris/HCl pH 8.0, 10 mM $MgCl_2$, 10 mM NaCl, 10 mM NaF, and 0.25 mM ATP). The crude kinase preparation was adjusted to a Cyt *f* concentration of 0.1 μg/μl quantified according to the method described in [28]. Either the reconstituted LHCII directly harvested from a sucrose density gradient, or the isolated authentic LHCII, was added to the phosphorylation assay mixture, to a final Chl concentration of 0.25 μg/μl. The mixture was incubated under a light irradiance of 100 μmol photons $m^{-2}s^{-1}$ for the indicated times. The phosphorylation was terminated with phosphorylation stop buffer (1.5% Tris/HCl pH 8.0, 4% SDS, 40% glycerol, 10% 2-mercaptoethanol).

B: Phosphorylation of recombinant Lhcb proteins with thylakoid membranes: The *in vitro* phosphorylation reaction was carried out in reaction mixture (100 μl) containing the reaction buffer (25 mM Tricine–NaOH (pH 8.0)/100 mM Sorbitol/20 mM NaCl/5 mM MgCl2/0.2 mM ATP/10 mM NaF), the reconstituted LHCII equivalent to 0.1 μg Chl, and the thylakoid membrane extract (1.5 mg/ml Chl). Unless otherwise specified, all incubations were carried out at 25 °C under a light irradiance of 100 μmol photons $m^{-2}s^{-1}$. Phosphorylation was terminated by addition of the phosphorylation stop buffer.

2.7. Chlorophyll quantification

For Chl quantification, 3 optical densities (wavelength at 750 nm, 663.6 nm, and 646.6 nm) of Chls dissolved in 80% acetone were measured with a Shimadzu UV–VIS 2550 spectrophotometer (Shimadzu, Inc., Japan) and the concentrations were calculated based on the three optical density measurement with the method described in [29].

2.8. Electrophoresis and immunoblot analysis

For analyzing the Lhcb phosphorylation, the same amounts of phosphorylation reaction mixtures (based on equal amount of Chl) were subjected to SDS polyacrylamide (15%) gel electrophoresis (PAGE) according to the method of [30]. After electrophoresis, the gels were either stained with Coomassie Brilliant Blue G-250 (CBB), or further subjected to immunoblot analysis. For the latter analysis, the proteins were transferred electrophoretically to nitrocellulose membrane (General Electric, Inc., USA). The membranes were blocked with bovine albumin at room temperature for 60 min and immunoblotted with antibodies against phosphothreonine (Cell Signaling Technology, Inc., USA), Lhcb1, or Lhcb2, (Agrisera, Inc., Sweden) according to standard procedures. The DyLight™ 800 labeled secondary antibody (Kirkegaard & Perry Laboratories, Inc., USA) was detected by scanning for infrared signals using the Odyssey Infrared Imaging System (LI-COR Biosciences, Inc., USA) according to the supplier's recommendations.

2.9. Molecular docking

The structure of Stt7d from *Micromonas* (4IX6 crystal structure [31] was used for the molecular docking analysis to gain insights into the interaction between LHCII kinase and its substrate. The AutoDock 4.2 program [32] was used to dock the peptide substrate into the STN7 structure. AutoDock Tools (version 1.5.6) was used to analyze the docking results. The STN7 macro-molecular structure was obtained by subtly adjusting the structure of Stt7d). Some of the residues of Stt7 in the catalytic domain were substituted with those from STN7, in a way that did not significantly alter the conformation of Stt7. The substrate ligand of LHCII (RKSATTKK) obtained from the sequence of Lhcb1 from pea was artificially put into the cave between the two lobes of the catalytic domain of STN7. The binding energy between STN7 and the ligand in these artificial complexes was estimated using the AutoDock program.

3. Results

3.1. The crude thylakoid kinase preparation contains several protein kinases, and phosphorylates reconstituted Lhcb1, but not Lhcb2

Plants were pretreated with far-red light (PSI light) for 3 h prior to the LL treatment so that all the antenna proteins were dephosphorylated. These plants were subjected to LL, and the phosphorylation kinetics of Lhcb1 and Lhcb2 *in vivo* were determined (Fig. 1). Lhcb2 was clearly phosphorylated faster than Lhcb1, which confirms earlier observations in *Arabidopsis*[17] and spinach [16]. The differences in phosphorylation kinetics imply that the underlying mechanisms of substrate recognition are different. In order to elucidate the different substrate specificities of the LHCII kinase towards Lhcb1 and Lhcb2, a crude kinase extract was prepared from spinach thylakoids with the method of Zer *et al.*[26]. Immunoblot analysis demonstrated that the crude kinase preparation contained STN7 kinase (Fig. 2A). To determine the global kinase composition of the crude kinase preparation, the gel slices from 25 kDa to 100 kDa were cut and subjected to LC-MS/MS analysis. Table S2 shows that 13 different kinases could be identified, in particular 7 Ser/Thr protein kinases including the known kinases STN7 and STN8. The phosphorylation kinetics of Lhcb1 with the crude kinase preparation showed that maximum activity was reached after *ca.* 20 min (Fig. 2B). This phosphorylation reaction was examined under different conditions. Firstly, the kinase activity was not strongly influenced by the DDM concentration (Fig. 2E). Furthermore, the pH sensitivity of the crude thylakoid kinase preparation was examined between pH 5 and 9 (Fig. 2C). The results indicate that the kinase extract was active over a relatively wide pH range. The reconstituted Lhcb1 (reLhcb1) could be phosphorylated by the crude kinase preparation when the pH was higher than 6, and reached its maximum at pH 9. Interestingly, the crude thylakoid kinase preparation could not phosphorylate reconstituted Lhcb2 *in vitro* (Fig. 2C; F). Phosphorylation assays with Lhcb2 over the whole pH range showed no activity, neither at the level of P-Thr or P-Ser (data not shown). Fig. 2F shows the results of phosphorylation with LHCII heterotrimers composed of Lhcb1, Lhcb2, and Lhcb3 monomers at different ratios. Only LHCII heterotrimers containing Lhcb1 were phosphorylated. Further analysis revealed that only the Lhcb1 subunit in the LHCII heterotrimer was phosphorylated. Hence Lhcb2 protein could not be phosphorylated by the crude kinase preparation, not only as homotrimer, but also as heterotrimer when trimerized with Lhcb1 and/or Lhcb3. Since the phosphorylation of the recombinant pea Lhcbs was catalyzed by a spinach kinase extract, further experiments were conducted with a kinase extract from the same plant species, pea (Fig. 2G). The results clearly show that the kinase isolated from pea behaves almost exactly as the kinase from spinach. In particular, both extracts could not catalyze the phosphorylation of reLhcb2 under all the tested pH conditions. This result strongly implies that different substrate recognition mechanisms operate for Lhcb1 and

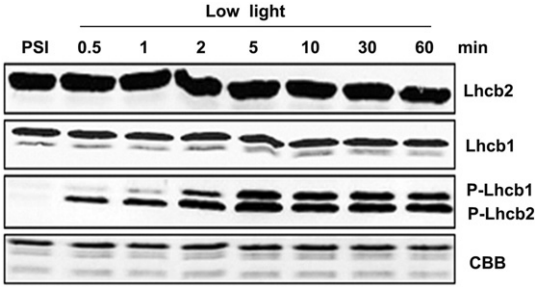

Fig. 1. Phosphorylation kinetics of Lhcb1 and Lhcb2 with pea thylakoids under low light (LL) (30 μmol photons m^{-2} s^{-1}). The plants were pretreated with far-red light (PSI light) for 3 h, and subsequently subjected to LL. The thylakoid membranes were isolated and solubilized in SDS, and then subjected to Tris-SDS-PAGE (15% Acr-Bis (37.5:1)) and immunoblotted with phosphothreonine antibodies. CBB indicates Coomassie blue staining as control for equal loading.

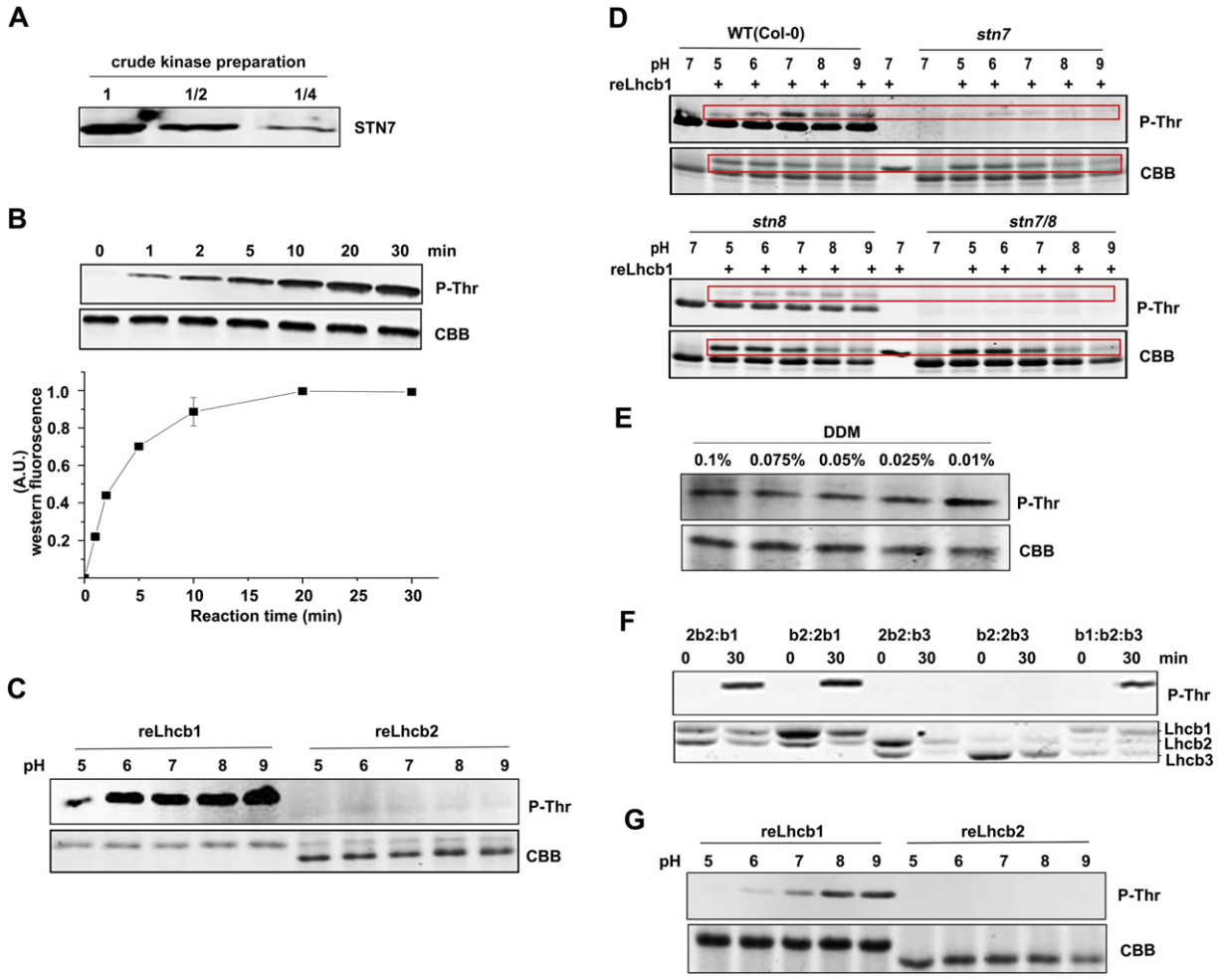

Fig. 2. Analysis of the crude thylakoid kinase preparation. (A) Immunoblot analysis of the crude thylakoid kinase preparation from spinach with STN7 antibody. Samples corresponding to 1, 0.5, and 0.25 μg Cyt *f* protein were loaded (lane 1, 1/2, 1/4); (B) kinase assay of the crude thylakoid kinase preparation from spinach with recombinant LHCII homotrimers composed of Lhcb1 as the substrate; (C) phosphorylation of recombinant LHCII homotrimers composed of either Lhcb1 or Lhcb2 at different pH conditions; (D) phosphorylation assay of reconstituted Lhcb1 incubated with thylakoid membranes from *Arabidopsis* mutants with different pH conditions; the added recombinant Lhcb1 proteins are framed in red; (E) In vitro phosphorylation analysis of recombinant LHCII from pea by the crude thylakoid kinase extract in a reaction buffer containing different concentration of DDM (N-Dodecyl β-D-Maltoside); (F) phosphorylation assay of recombinant LHCII heterotrimers containing combinations of Lhcb1/2/3 at different ratios; (G) phosphorylation of recombinant LHCII homotrimers composed of either Lhcb1 or Lhcb2 with a pea kinase extract at different pH conditions. The LHCII protein before (0 min) and 30 min after the phosphorylation reaction (30 min) were subjected to Tris-SDS-PAGE and immunoblot analysis using phosphothreonine antibodies.

Lhcb2. In order to examine the importance of the amino acid residues in Lhcb1 and Lhcb2 responsible for these differences, site-directed mutagenesis was performed on both Lhcb1 and Lhcb2. After reconstitution of protein pigment complexes, the modified proteins were tested using the phosphorylation assay described above.

As shown in Table S2, the crude kinase extract contains multiple kinases. To elucidate whether the STN7 and STN8 kinases are involved in the specific recognition of reLhcb1/2, thylakoid membranes isolated from different *Arabidopsis* strains, namely WT and mutants *stn7*, *stn8 and stn7/8*, were used for phosphorylation of reLhcb1/2 over a pH range from 5 to 9 (Fig. 2D). The results indicate that reLhcb1 protein was phosphorylated when incubated with thylakoid membranes from the WT and *stn8* mutant. In contrast no phosphorylation of Lhcb1 was detected with extracts from *stn7 and stn7/8*. Therefore, it is very likely that the phosphorylation of reLhcb1 with the kinase extracts is largely dependent on the STN7 kinase.

3.2. The substrate specificity of Lhcb1 and Lhcb2 by kinases from Chlamydomonas reinhardtii

Although the activation loop of the STN7 kinase is conserved amongst the other orthologs of this kinase, it was important to show that the differences in phosphorylation of Lhcb1 and Lhcb2 of pea observed with the kinase extracts from pea and spinach are also observed with thylakoid kinase extracts from other species. We therefore prepared extracts from *C. reinhardtii* and performed *in vitro* phosphorylation assays with Lhcb1 and Lhcb2 from pea (Fig. 3). The results show that LHCII phosphorylation is strongly dependent on the detergent concentration with an optimum at 0.02% DDM. Moreover, the kinase extract from *C. reinhardtii* could phosphorylate only reLhcb1, but not reLhcb2, based on the different migrations of reLhcb1 and reLhcb2 compared with the native LHCIIs in the thylakoid membrane (Fig. S1). This result indicates clearly that the kinase from *C. reinhardtii* has the same specificity for the phosphorylation of Lhcb1 and Lhcb2 as the kinase from spinach/pea.

3.3. The positively charged residues at the N-terminal of the Lhcb1 are critical for its phosphorylation

Since the sequence analysis of Stt7/STN7 protein kinase clearly identified several negatively charged residues in the activation loop (Fig. 4A), we assessed the role of the key conserved positively charged residues (R/K) in the vicinity of the phosphorylation site in the N-terminal domain of Lhcb1 for the phosphorylation activity. Four residues in the N-terminal

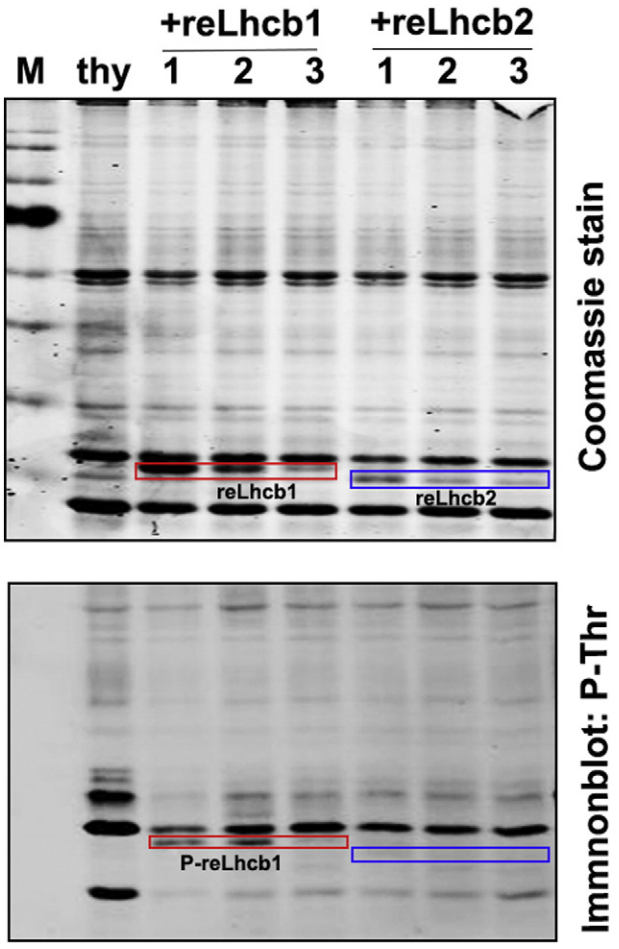

Fig. 3. Analysis of the phosphorylation specificity of recombinant pea Lhcb1/2 with thylakoid kinase extracts from *Chlamydomonas reinhardtii* thylakoid. Samples corresponding to different volume ratios of reLhcb/thylakoid: 1/2, 1/5 and 1/10, also corresponding to different DDM concentration: 0.05%, 0.02%, and 0.01% DDM, respectively in the reaction assay, were loaded (lane 1, 2, 3). The red/blue rectangles frame the added reLhcb1/reLhcb2 proteins. Thylakoid membrane proteins stained with Coomassie blue (top); the phosphorylated proteins were identified by immunoblot analysis with anti-P-Thr antibodies (bottom).

domain of Lhcb1, namely R1, K2, K7, K8, were selected for site-directed mutagenesis and subsequent phosphorylation assays (Fig. 4B). Five Lhcb1 mutants including four with a single amino acid change—(Lhcb1-R1A, Lhcb1-K2A, Lhcb1-K7A and Lhcb1-K8A), and one with two amino acid changes (Lhcb1-R1K2AA) were produced. The phosphorylation activities of all the mutants were either diminished or abolished. Lhcb1 phosphorylation was eliminated in the mutants with the changes R1A and K7A. TheLhcb1-K2A mutant maintained approximately 10% phosphorylation, and Lhcb1-K8A, about 40%. The mutant with the double-residue change showed no phosphorylation at all (Fig. 4C). These results indicate that the positively charged residues close to the phosphorylated site (Thr) are crucial for the phosphorylation activity.

3.4. The N-terminal sequence upstream of the phosphorylation site (Thr) in Lhcbs is important for Lhcb phosphorylation

Comparison of the N-terminal polypeptide sequences of Lhcb1 and Lhcb2 from pea is shown in Fig. S2. The sequences are highly conserved, except for the first 12–15 residues near the N-terminus, which displays remarkable variations. The alignment shows that the sequences upstream of the phosphorylation site (Thr) are different in Lhcb1 and Lhcb2. There are four residues before the phosphorylation site (Thr-5) in Lhcb1, but only two in Lhcb2 (Thr-3). Since the sequence upstream of the phosphorylation site (Thr) is located close to the interaction motif of Lhcb with the kinase, as proposed by [24], we tested whether the different N-terminal sequences of the two Lhcbs account for the differences in phosphorylation. By exchanging the N-terminal sequence of Lhcb1 with that of Lhcb2, we generated a Lhcb1 variant with the N-terminal sequence of Lhcb2 (Lhcb1-N2) and in a reciprocal manner a Lhcb2 variant with the N-terminal sequence of Lhcb1 (Lhcb2-N1). The Lhcb2-N1 peptide had four amino acid residues (RKSA) upstream of the phosphorylation site (Thr-5), while that of the Lhcb1-N2 contained only two residues (RR). The results of the phosphorylation assay of the two mutants clearly showed that substituting the N-terminal sequence of Lhcb2 with that of the Lhcb1 conferred the phosphorylation properties of Lhcb1, since the phosphorylation activity to Lhcb2-N1 was the same as that to Lhcb1 (Fig. 5). In a reciprocal way, replacement of the N-terminal sequence of Lhcb1 by that of Lhcb2 abrogated the phosphorylation. These results indicate that the different phosphorylation activities of Lhcb1 and Lhcb2 from pea can be accounted for by their different N-terminal sequences upstream of the phosphorylation site. The N-terminal stretches are critical for the recognition by the kinase and for the phosphorylation.

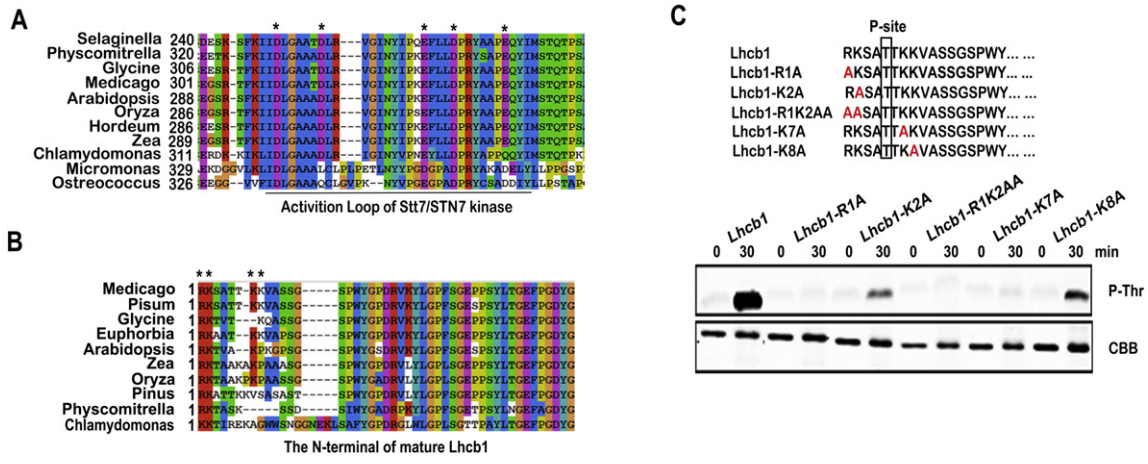

Fig. 4. Importance of the positively charged amino acids at the N-terminal end of Lhcb1. (A) Alignment of the activation loop domains of various Stt7/STN7 kinase proteins; (B) alignment of the N-terminal sequence of mature Lhcb1; multiple sequence alignment was performed with clustal X2. The conserved negatively charged amino acids in the kinase and the positively charged residues in Lhcb1 are indicated with asterisks; (C) immunoblot analysis with anti-phosphothreonine antibodies of the recombinant Lhcb1 homotrimers and its different mutants after the phosphorylation assay (The graphic above shows the modified sites on Lhcb1 highlighted with red color). Abbreviations: Arabidopsis, *Arabidopsis thaliana*; Chlamydomonas, *Chlamydomonas reinhardtii*; Euphorbia, *Euphorbia esula*; Glycine, *Glycine max*; Hordeum, *Hordeum vulgare*; Medicago, *Medicago sativa*; Micromonas, *Micromonas Algae*; Oryza, *Oryza sativa Japonica Group*; Ostreococcus, *Ostreococcus lucimarinus*; Physcomitrella, *Physcomitrella patens*; Pinus, *Pinus thunbergii*; Pisum, *Pisum sativum*; Spinacia, *Spinacia oleracea*; Zea, *Zea mays*.

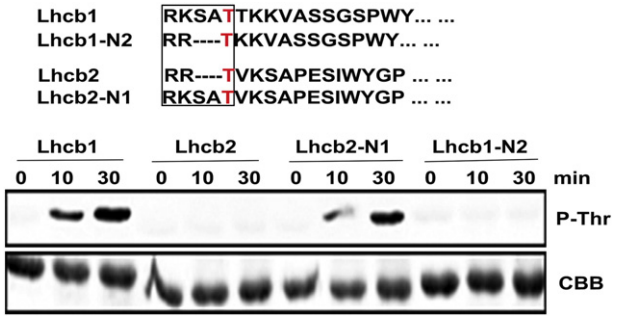

Fig. 5. Phosphorylation of the LHCII homotrimers composed of either Lhcb1 or Lhcb2 and their mutant forms with exchanged N-termini. Lhcb1-N2: Lhcb1 with the starting sequence of Lhcb2, and Lhcb2-N1: Lhcb2 with the starting sequence of Lhcb1 (the potential phosphorylation site of Lhcb protein is highlighted in red color).

3.5. Identification of the key motifs for the phosphorylation activity in the N-terminal sequence upstream of the phosphorylation site in Lhcb1

In order to investigate the basic sequence requirement for the phosphorylation of Lhcb1 by the kinase, further mutagenesis studies were carried out on the N-terminal sequence upstream of the phosphorylation site (Thr-5) in Lhcb1. The mutagenesis was designed to study the effect of the length and of the amino acid composition of the N-terminal sequence upstream of the phosphorylation site on the phosphorylation activity. Nine Lhcb1 mutants were obtained by deleting one or two residues from the N-terminal sequence upstream of the phosphorylation site in Lhcb1, including five mutants with single-residue deleted proteins (Lhcb1-R, Lhcb1-K, Lhcb1-S, Lhcb1-A, Lhcb1-T) and four mutants with two residue-deleted proteins (Lhcb1-RK, Lhcb1-KS, Lhcb1-SA, Lhcb1-TT). These Lhcb1 derivatives were reconstituted with thylakoid pigments, and subjected to the phosphorylation assay. Fig. 6A shows the positions of the deleted residues of different mutants and their phosphorylation patterns. Mutants Lhcb1-R, Lhcb1-SA, Lhcb1-TT, and Lhcb1-RK had reduced phosphorylation activities compared with the WT Lhcb1. A complete loss of phosphorylation was observed for the double-residue deletion mutants (Lhcb1-RK and Lhcb1-TT) (Fig. 6A), revealing the importance of the positively charged residues for kinase recognition, in agreement with the results in Fig. 4. Other mutants showed diverse results. The SA double deletion (Lhcb-SA) decreased the phosphorylation by 70%, although this effect was not as extensive as that of the Lhcbs with two positively charged residues deleted, while deleting the T/A residue led to an increased Lhcb1 phosphorylation. Interestingly, removal of K/S residues in the N-terminal sequence had no adverse effect on phosphorylation. Moreover, deleting the residues KS, with the N-terminal sequence starting with RATT, resulted in even higher phosphorylation. In order to further clarify the role of specific amino acids with regard to phosphorylation, additional mutagenesis was carried out with the mutant Lhcb1-KS, with either the second residue A from the N-terminal in Lhcb1-KS changed (Lhcb1-RRTT, —RKTT, and -RWTT), or the second potential phosphorylation site (Thr-4) substituted by an A (Lhcb1-RATA). The results clearly show that substituting the A with a positively charged residue (R/K) completely eliminated the phosphorylation (Fig. 6B). Exchanging A to W retained only about 1/5 phosphorylation activity. Fig.6B also shows that changing the second potential phosphorylation site (Lhcb1-RATA) resulted in a complete loss of phosphorylation, suggesting that the residue after the phosphorylation site affected the recognition of the kinase. Alternatively, this result might also imply that the phosphorylation site in the Lhcb1-KS is the second T, rather than the first one, which indicates the necessity of a certain minimal length before the phosphorylation site for the kinase–Lhcb1 recognition. Additional mutagenesis studies also verified the requirement of a certain minimal length before the phosphorylation site. Fig. 6C shows that deleting T6 resulted in the complete loss of the phosphorylation activity. There are two Thr residues in the N-terminal domain of Lhcb1 and our results suggest that both sites

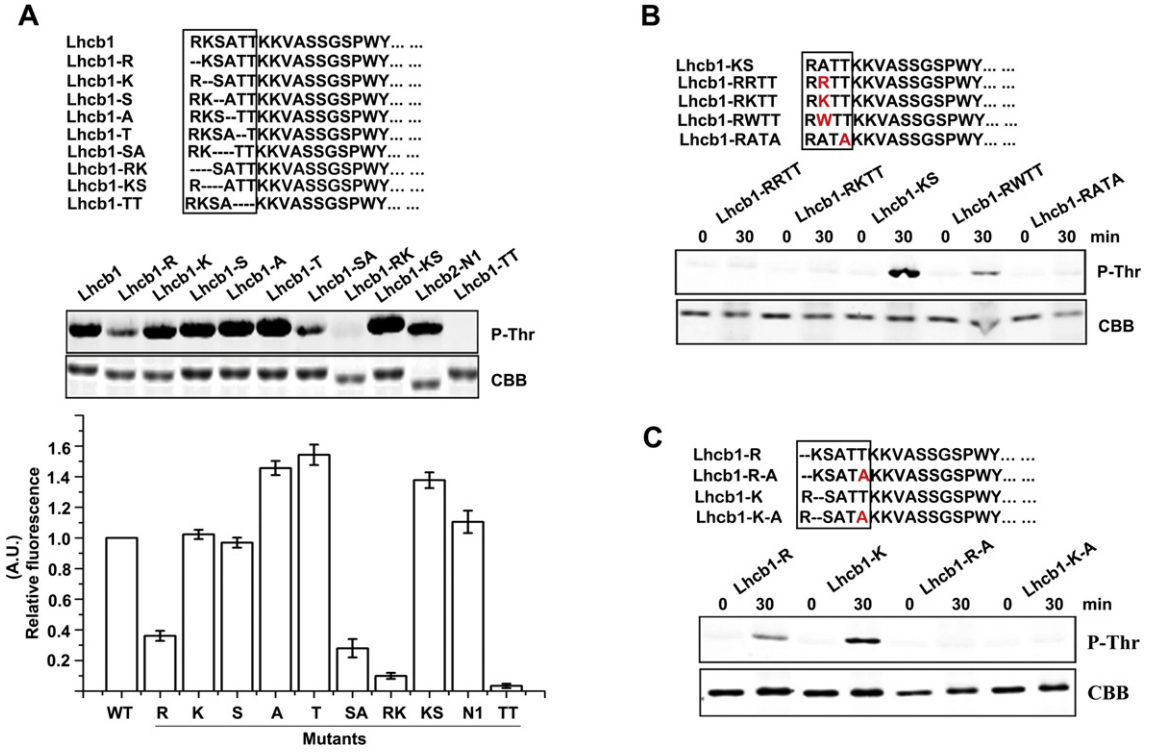

Fig. 6. Phosphorylation assay of the WT Lhcb1 homotrimers and its different N-terminal sequence mutants. (A) Comparison of the phosphorylation activities of the WT Lhcb1 trimers and of the mutant LHCII homotrimers composed of Lhcb1 with one or two residues deleted in the N-terminal sequence. The bottom graphic shows the relative quantification of the phosphorylation signals. The X-axis presents the Lhcb mutants: The letters indicate the amino acid deleted from the N-terminal end, and N1 means the N-terminal end of Lhcb2 exchanged with that of Lhcb1; (B) effects of the second round mutagenesis on the phosphorylation activity (enhanced mutant Lhcb1-KS); (C) identification of the key sites in the N-terminal sequence preceding the phosphorylation site for an active Lhcb phosphorylation. The mutated sites of Lhcbs are framed and colored in red.

can be phosphorylated, depending on the sequence preceding the phosphorylation site. Shortening the N-terminal sequence by one amino acid may move the phosphorylation site to the second Thr.

In summary, both the positively charged amino acid R/K at the N-terminus of Lhcb1 and the number of the residues upstream of the phosphorylation site are essential for Lhcb1 phosphorylation.

3.6. Requirement for a unique length of the N-terminal sequence preceding the phosphorylation site of Lhcb2

Unlike Lhcb1, which possesses two potential phosphorylation sites and four residues before the first potential phosphorylation site, Lhcb2 contains only one Thr and two residues before the phosphorylation site. In order to confirm the proposed structural requirement for Lhcb1 phosphorylation, site directed mutagenesis was performed on the N-terminal domain of Lhcb2 by inserting one or two Ala residues before the phosphorylation site, so that the length of the N-terminal sequence upstream of the phosphorylation site was increased. Two mutants with elongated N-terminal sequence (Lhcb2-RRA and Lhcb2-RRAA) were generated and subjected to the phosphorylation assay (Fig. 7A).The results indicate that Lhcb2 was strongly phosphorylated in the Lhcb2-RRAA mutant (Fig. 7A). Moreover, four Lhcb2 mutants with one of the residue in the first two amino acids exchanged (Lhcb2-RK, Lhcb2-KK, Lhcb2-KR and Lhcb2-RW) were all deficient in Lhcb2 phosphorylation. It thus appears that both the length and the sequence of the peptide before the phosphorylation site determine the substrate–kinase recognition.

In both Lhcb1 and Lhcb2, the first two residues are positively charged with RK for Lhcb1 and RR for Lhcb2. The role of these residues was investigated by site-directed mutagenesis. To the elongated Lhcb2 derivative (Lhcb2-RRAA), the second residue "R" was changed to "K", so that the peptide begins with the same two amino acids as Lhcb1, and *vice versa* the second residue "K" in Lhcb1 was changed to "R", so that the Lhcb1 mutant begins with the same two amino acids as Lhcb2. Phosphorylation activity assays with these different reconstituted Lhcb mutants revealed interestingly that the two N-terminal RR residues might be responsible, at least in part, for the faster phosphorylation kinetics of Lhcb2 because the RR-Lhcb2 mutant gave rise to a faster and higher phosphorylation than the RK Lhcb2 mutant *in vitro* (Fig. 7B). It is noticeable that the changes of the N-terminal residues RR in Lhcb1 also resulted in increased phosphorylation compared to the WTRK-Lhcb1 (Fig. 7B).

4. Discussion

It is well known that the structures of the catalytic sites of most eukaryotic protein kinases are similar. Classical protein kinases have a canonical catalytic domain containing *ca.* 250 residues [33,34], differing in terms of the charge and hydrophobicity of surface residues that are important for kinase specificity [35–37]. Previous studies have demonstrated that the activation loop might adopt various conformations in the inactive and active states with an important impact on the control of kinase activity [31,38,39]. Previous work has revealed that the phosphorylation of Lhcb1/2 follows different kinetics as Lhcb2 is phosphorylated significantly faster than Lhcb1 [40]. Furthermore, it was proposed that the phosphorylation of Lhcb1/2 plays complementary roles in state transitions [21]. In this work, all the *in vitro* phosphorylation assays of the reconstituted pea Lhcb1/2 with kinases from various species, namely pea, spinach and *C. reinhardtii* showed that Lhcb1, but not Lhcb2 could be phosphorylated, and that the N-terminal sequence of Lhcbs is critical for the LHCII kinase recognition (Fig. 3). Moreover, phosphorylation activities obtained with the kinase extracts from pea and spinach with specific Lhcb mutant forms (Lhcb2-N1, Lhcb1-N2, Lhcb2-AA and Lhcb1-R1A) give rise to the same results (Fig. S4). It can be concluded that the recognition of Lhcb1 and Lhcb2 by the LHCII kinase is conserved in different species.

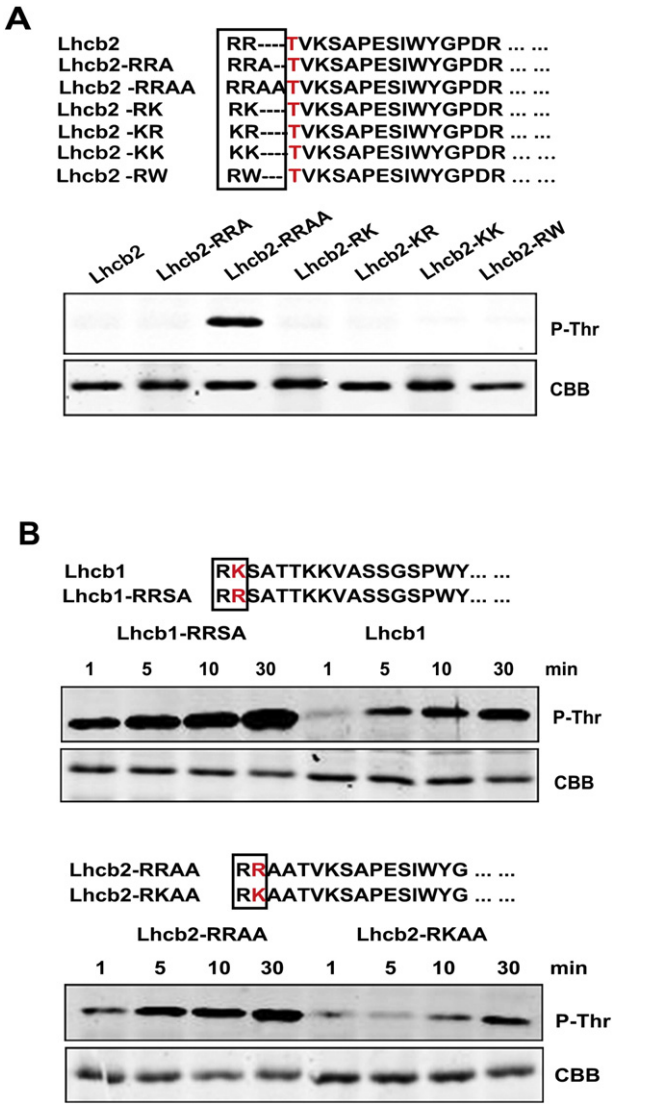

Fig. 7. Phosphorylation assay of different Lhcb mutants: (A) Immunoblot analysis of the phosphorylation activity of Lhcb2 mutants. The mutated sites are framed. The possible phosphorylation sites are in red letters; (B) immunoblot analysis of Lhcb1 and Lhcb2 mutants with changes in the first two amino acids with P-Thr antibodies. The proteins used are WT Lhcb1/2, and different mutants (Lhcb1 with starting sequence of Lhcb2 (RR) and Lhcb2 with the starting sequence of Lhcb1 (RK)). The residues of interest are framed, and the key residues are red colored. Phosphorylation assays were stopped after 1, 5, 10, and 30 min.

4.1. Phosphorylation of Lhcb1 and Lhcb2 could occur through different mechanisms

The Lhcb2 phosphorylation was observed to be significantly faster than that of Lhcb1 in pea leaves incubated under LL (Fig. 1). Our *in vivo* experiments confirm previous studies on different species, that Lhcb2 is phosphorylated faster than Lhcb1 [16,17,21,41]. However, in contrast to the *in vivo* observations, the recombinant Lhcb2 from pea could not be phosphorylated by the crude kinase preparation in our *in vitro* phosphorylation assay (Fig. 2C, G). In contrast this kinase preparation could phosphorylate Lhcb1 under the same conditions. These differences in phosphorylation of Lhcb2 or Lhcb1 observed *in vitro* strongly suggest that the different antenna proteins (Lhcb1 and Lhcb2) interact differently with the kinase. It is not clear why Lhcb2 could not be phosphorylated *in vitro* although it is phosphorylated faster than Lhcb1 *in vivo*. Multiple factors contribute to the substrate specificity, including the structure of the catalytic cleft in the kinase [42,43], local and distal interactions between the kinase and substrate (consensus sequences [44],distal

docking sites [45]), targeting subunits [46,47], localization of the kinase [48,49], scaffolds [50,51], and systems-level effects [52,53]. A reasonable hypothesis is that the STN7 kinase qualifies as the LHCII kinase although a direct proof is still lacking and that this kinase must interact with Lhcb1 and Lhcb2 [17]. In our phosphorylation assay system the interaction between STN7 and Lhcb2 appears to be compromised, which prevents the phosphorylation of Lhcb2. Furthermore, the analysis of site directed Lhcb mutants revealed the importance of the N-terminal sequence preceding the phosphorylation site for the kinase–substrate recognition. Exchanging the four residues at the N-terminal of Lhcb2 with those of Lhcb1 (Fig. 5), or increasing the N-terminal peptide to a length similar to that of Lhcb1 (Fig. 7), resulted in Lhcb2 phosphorylation. This suggests that the inability to phosphorylate Lhcb2 *in vitro* could be attributed to the difference in the N-terminal sequence of Lhcb2 compared with that of Lhcb1. It is probable that the conditions for STN7 kinase activity differ in the *in vitro* phosphorylation assay and that this prevents phosphorylation of Lhcb2.

It is known that the N-terminal sequence upstream of the phosphorylation site plays important roles in regulating antenna protein phosphorylation. Our mutagenesis analysis demonstrates that not only the positively charged residues, but also the length of the peptide upstream of the phosphorylation site is very important for activating the phosphorylation of Lhcb proteins. Our mutagenesis study further suggests that the N-terminal amino acid sequences of Lhcb are also critical for regulating the rate of protein phosphorylation. Exchanging the first two residues of Lhcb1 and extending the Lhcb2 N-terminal region revealed that the RR residue containing peptide is phosphorylated faster *in vitro* (Fig. 7B). Since RR is very much conserved in Lhcb2 (Fig. S3), the two consecutive RR might play a special role for the faster kinetics of Lhcb2 phosphorylation *in vivo*.

Similar to Lhcb1, the Lhcb2 protein possesses a conserved phosphorylation site Thr-3 at the N-terminal end (Fig. S3), which is also surrounded by positively charged amino acids (R) [18]. Compared with Lhcb1, the N-terminal domain of Lhcb2 from different species is more conserved, which may hint to an important role of this Lhcb2 region [21]. Several features of Lhcb2 support this proposal because of its specific peripheral localization in the thylakoid membrane and its structural stability compared to the other LHCII isoforms. Lhcb2 is the first phosphorylated protein upon light exposure as observed here (Fig. 1) and in several other reports [16,17]. Lhcb2 undergoes structural variation mostly via phosphorylation [16,54]. It is located in a peripheral position of PSII particles[40], which correlates with the finding that phosphorylated Lhcb2 is mainly associated with PSI [21]. Lhcb2 also has been shown to have unique structural properties such as the lowest stability at elevated temperature and under strong light irradiance [24]. It has been suggested that Lhcb1 and Lhcb2 play individual but complementary roles during state transitions because the *in vivo* Lhcb2 phosphorylation is much faster than that of Lhcb1, while Lhcb1 is more phosphorylated during state transitions [17]. The function of Lhcb2 in state transitions appears to trigger the phosphorylation and the migration of LHCII trimers to PSI, while that of Lhcb1 functions, in coordination with Lhcb2, in sorting the thylakoid membrane, as is required for the antenna phosphorylation and the migration of the phosphorylated proteins [21,40,41]. Phosphorylated Lhcb1 were found either in PSI or in PSII, or in a new LHCII mega-complex, which might be related to the heptameric association of LHCII observed by electron microscopy [17,21,55,56].

4.2. Implications for the interaction of the LHCII kinase with their peptide substrates

Characterization of the structural/functional properties of Stt7/STN7 kinases has been difficult. In an effort to crystallize the kinase–substrate complex, only one clone from the more than 50 tested clones of *Micromonas sp.* RCC299 could be crystallized but no peptide substrates were found in the resolved structures, either by soaking the MsStt7d-KD crystals in a solution with excess peptide substrates or by co-crystallizing the MsStt7d-KD with peptide substrates [31]. Our studies reveal that the length of the peptide chain preceding the phosphorylation site (Thr) plays a crucial role for phosphorylation, as revealed in Fig. 5 and Fig. 6B. It was confirmed by the mutagenesis study of Lhcb2, because adding or deleting only one amino acid to the N-terminal sequence preceding the phosphorylation site resulted in exactly the reverse phosphorylation effect (Fig. 7A). Lhcb1 protein from pea contains two Thr residues in the N-terminal hydrophilic domain, one of which (Thr-5) was identified as the sole phosphorylation site (22). With our truncated mutants of Lhcb1, we demonstrated that the Thr-6 could also be phosphorylated when the length of the peptide upstream of the phosphorylation site was shortened (Fig. 6C). These observations confirm the importance of the peptide length preceding the phosphorylation site at the N-terminal end of Lhcb for the substrate recognition by the kinase. Therefore, our study provides useful information for inferring the molecular architecture of Stt7/STN7 kinase domain and to gain insights into its catalytic and regulatory mechanisms through the high-resolution crystal structures of the kinase in a complex with proper substrates.

According to the multiple sequence alignment of the thylakoid protein kinase domains of various Stt7/STN7 family members, the thylakoid protein kinases Stt7/STN7, which belong to the classical Ser/Thr protein kinase family with a canonical protein kinase fold that can be divided into two lobes, contain five negatively charged E/D residues in the activation loop region. Based on the mutagenesis analysis, we found that replacing the positively charged residue (Arg) of Lhcb1 with a neutral amino acid (Ala) disrupted the phosphorylation activity of Lhcb1 (Fig. 3), which suggests that this residue plays an important role in the recognition and/or interaction of Lhcb1 and the kinase.

In order to gain insights into the peptide/protein substrate binding site of the STN7 kinase, a molecular simulation was conducted. Since LHCII kinase belong to the Ser/Thr protein kinase family, the crystal structure of Stt7d of *Micromonas* 4IX6 [31] of the same kinase family was used for the analysis of the LHCII kinase–Lhcb1 interaction (Fig. 8). Since neither the sequence of the STN7 kinase from pea, and spinach are available, the kinase sequence from *Arabidopsis* was used for a molecular simulation The BLAST in PDB database revealed that the STN7 sequence from *Arabidopsis* showed the highest sequence identity with Stt7d from *Micromonas* algae. Based on this analysis, the macromolecular structure of STN7 was modulated by adjusting the Stt7d structure (4IX6) based on 4IX6 and the STN7 sequence. The substrate ligand of LHCII (RKSATTKK) was docked into the inner pocket of STN7 with different conformations. The conformation with the lowest binding energy between STN7 (4.11 kcal/mol) and ligand was chosen for the docking model to describe the interaction between STN7 and the substrate. This analysis revealed that the potential binding site for the Lhcb1 substrate on the kinase was around the flat surface area between the glycine-rich loop in the N-terminal lobe and the activation loop in the C-terminal region, where a negative electrostatic potential patch was found to target the substrate. The results are compatible with our biochemical data revealing that the positively charged amino acids (R/K) in the N-terminal domain near the phosphorylation site of Lhcb1 are essential for the substrate–kinase interaction with the negatively charged amino acids E/D in the activation loop of the Stt7/STN7 kinase.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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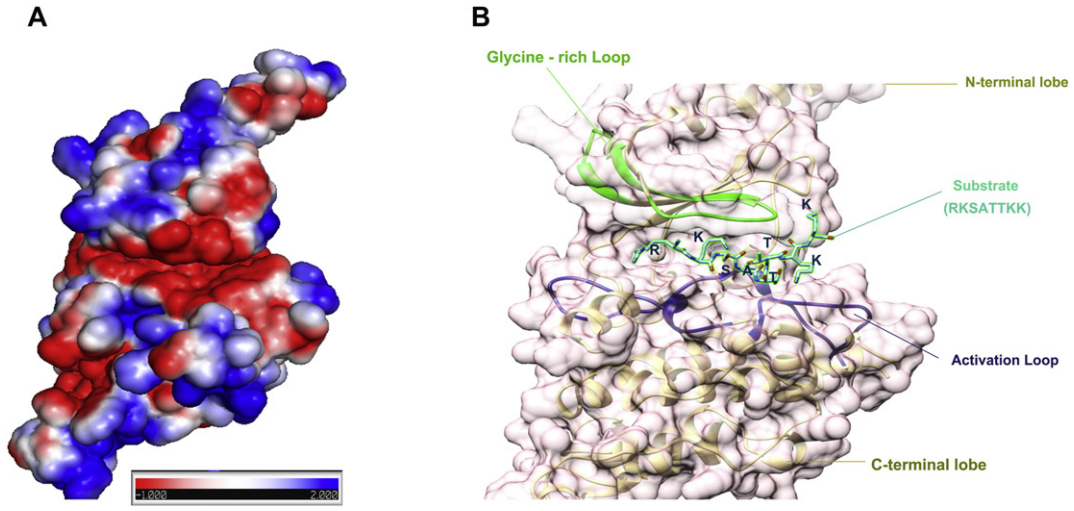

Fig. 8. In silico analysis of surface of the STN7 kinase reveals a negatively charged potential patch as a target for binding the substrate(s). (A) The AtSTN7 structure is shown as electrostatic potential surface model with calculated electrostatic potential: deep red indicates electronegative and deep blue indicates electropositive domains, respectively; (B) structure of the AtSTN7 kinase in a complex with the peptide substrate (RKSATTKK) using the Autodock program.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2015.10.012.

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