Received Date : 14-Jun-2016
Revised Date : 24-Oct-2016
Accepted Date : 28-Oct-2016
Article type : Original Article

The STN8 kinase-PBCP phosphatase system is responsible for high-light-induced reversible phosphorylation of the PSII inner antenna subunit CP29 in rice

Nico Betterle[1,3,†], Roshan Sharma Poudyal[2,4,†], Anthony Rosa[1,†], Guangxi Wu[2], Roberto Bassi[1,*] and Choon-Hwan Lee[2,*]

[1]*Dipartimento di Biotecnologie, Università di Verona, Ca' Vignal 1, Strada le Grazie 15, 37134 Verona, Italy*

[2]*Department of Molecular Biology, Pusan National University, Busan 609-735, Korea*

[†]equal contribution,

[*]co-corresponding authors (emails roberto.bassi@univr.it; chlee@pusan.ac.kr)

[3]*Current address: Department of Plant & Microbial Biology, University of California, Berkeley, 111 Koshland Hall, Berkeley, CA 94720-3102, USA*

[4]*Current address: Center for Plant Aging Research, Institute for Basic Science, Daegu 42988, Korea*

RUNNING TITLE: STN8 and PBCP are involved in CP29 phosphorylation

KEYWORDS: high-light illumination, protein phosphorylation, Photosystem II, CP29, STN8 kinase, PBCP phosphatase, rice

SUMMARY

Reversible phosphorylation of thylakoid light harvesting proteins is a mechanism to compensate for unbalanced excitation of PSI vs PSII under limiting light. In monocots, an additional phosphorylation event on the PSII antenna CP29 occurs upon exposure to excess light, enhancing resistance to light stress. Different from the case of the major LHCII antenna complex, the STN7 kinase and its related PPH1 phosphatase were proven not to be involved in CP29 phosphorylation, indicating that a different set of enzymes act in the high-light response. Here, we analyze a rice *stn8* mutant in which both PSII core proteins and CP29 phosphorylation are suppressed in high light, implying that STN8 is the kinase catalyzing this reaction. In order to identify the phosphatase involved, we produced a recombinant enzyme encoded by the rice ortholog of *At*PBCP, antagonist of *At*STN8, which catalyzes the dephosphorylation of PSII core proteins. The recombinant protein was active in dephosphorylating P-CP29. Based on these data, we propose that the activities of the *Os*STN8 kinase and the antagonistic *Os*PBCP phosphatase, in addition to being involved in the repair of photo-damaged PSII, are also responsible for the high light-dependent reversible phosphorylation of the inner antenna CP29.

INTRODUCTION

Plants are exposed to rapid changes of their environment resulting in abiotic-type stresses which account for most of the productivity loss in crop species and primarily target the photosynthetic apparatus (Navabpour et al., 2003). Indeed, the efficiency of mechanisms that regulate photosynthesis is critical for plant productivity due to their ability to minimize oxidative damage from over-excitation resulting from fluctuations in

light intensity and/or changes in spectral composition of light within canopies (Nelson and Ben-Shem, 2004).

State transitions (ST) and Non-Photochemical Quenching (NPQ) are two major mechanisms for the rapid and reversible acclimation of the photosynthetic electron transfer chain to changing light. Indeed, (i) ST operate in limiting light by balancing the excitation energy delivery to PSI vs PSII, allowing for optimal photon use and avoiding plastoquinone (PQ) over-reduction and photoinhibition (Rochaix et al., 2012); and (ii) NPQ, through its main component qE (de Bianchi et al., 2010; Niyogi and Truong, 2013; Horton et al., 1996), catalyzes the thermal dissipation of energy absorbed in excess. ST are inhibited in high light (Rintamaki et al., 1997), whereas excess light activates qE through thylakoid lumen acidification. This acidification occurs due to the inhibition of ATP synthase by lack of its ADP substrate, when the catalytic rate of the Calvin-Benson cycle is exceeded. Over-excitation of Photosystem II (PSII) vs Photosystem I (PSI) activates ST by reducing PQ (Allen and Staehelin, 1992) and promoting the phosphorylation of stroma-exposed threonine residues in trimeric LHCII by the STN7 kinase (Bellafiore et al., 2005; Rochaix et al., 2012; Betterle et al., 2015). In *Arabidopsis thaliana*, a fraction of the phosphorylated LHCII, enriched in LHCB2 (Pietrzykowska et al., 2014; Longoni et al., 2015; Crepin and Caffarri, 2015), diffuses from grana partitions, where it participates to the PSII antenna system, towards stroma-exposed membranes where it increases PSI antenna size (Bassi et al., 1988; Galka et al., 2012). The STN7 activity is inhibited in high light through the reduction of a sulphur bridge (Shapiguzov et al., 2016). Upon inactivation of the kinase, the PPH1/TAP38 phosphatase (Shapiguzov et al., 2010; Pribil et al., 2010) reverses the process.

Besides LHCII, other PSII subunits including the PSII core proteins CP43, D1 and D2, are phosphorylated and this is fundamental for the turnover of components damaged by oxidative stress (Aro et al., 1994). This second type of phosphorylation, which is not prevented by high light, is catalyzed by the STN8 kinase (Bonardi et al., 2005; Nath et al., 2013a) and reverted by PBCP phosphatase (Samol et al., 2012). An additional high light-dependent phosphorylation event is restricted to monocots and consists in phosphorylation of the CP29 subunit of PSII which connects the LHCII-M trimer to PSII core complex (Bergantino et al., 1995, 1998; Hwang et al., 2003; Caffarri et al., 2009; Liu et al., 2009; Chen et al., 2009; Pursiheimo et al., 2003). CP29 phosphorylation enhances NPQ (Mauro et al., 1997; Betterle et al., 2015) and is STN7-independent (Betterle et al., 2015) whereas the kinase/phosphatase gene products involved are yet unknown. The functional redundancy for PSII core protein phosphorylation between STN7 and STN8 kinases is reduced in rice with respect to Arabidopsis (Nath et al., 2013a), leading to the hypothesis that STN8 might be involved in CP29 phosphorylation.

In this report, we analyze wild-type (WT), *stn8* knock-out, STN8 overexpressor and *stn7* rice genotypes to show that STN8 is the kinase responsible for CP29 phosphorylation. We also identify the enzyme involved in CP29 dephosphorylation. These findings open the way to the transfer of said process from monocots to dicots with the aim of increasing crop resistance to abiotic stresses.

RESULTS

Previous work on CP29 phosphorylation showed that it was independent from STN7 (Betterle et al., 2015). Moreover, the early observation that high light (HL) enhanced CP29 phosphorylation rather than inhibiting it, suggested that STN8 might be the kinase involved in this activity.

In order to verify this hypothesis, we used the *stn8* rice mutant (Nath et al., 2013a), and compared its CP29 phosphorylation behavior to that of other mutant lines recently described (Nath et al., 2013a; Betterle et al., 2015). Mutant rice lines *stn7*, *stn8*, *pph1*, a complemented line overexpressing STN8 (OE-STN8) and their respective WT cultivars were either maintained in the dark (D) or exposed to excess light (HL, 1500 μmol photons m^{-2} s^{-1}, 28 °C) for 60 min, after which thylakoid membranes were purified and their polypeptides analyzed by SDS-PAGE followed by immunoblot using anti-CP29 antibody (Figure 1). Two bands were detected and the slow migrating one corresponded to phosphorylated CP29 (P-CP29) as previously described (Bergantino et al., 1995; Betterle et al., 2015; Hwang et al., 2003). The upper band was present in HL treated samples with a similar abundance, whereas it was absent in the sample from *stn8* despite HL treatment, implying that STN8 and not the STN7 kinase was involved in the formation of P-CP29 in rice.

Indeed, HL treatment (1500 μmol photons m^{-2} s^{-1}, 30 min 24 °C) induced the appearance of a new sharp Coomassie-stained band with an apparent molecular weight (MW) of 30 kDa in WT samples which was absent in the *stn8* genotype (Figure 2A). Immunoblot analysis using anti-CP29 antibody showed two bands of, respectively, 30 and 28 kDa in the sample from HL treated WT. In contrast, samples from dark-adapted (D) WT and from *stn8,* either D or HL only showed the low MW form of CP29. The

intensity of the 28 kDa band was decreased by ~50% in HL WT vs D as quantified from both the Coomassie-stained gel and the anti-CP29 antibody reaction.

Immunoblot with anti-Phosphothreonine (P-Thr) antibody on the same samples revealed three major bands at, respectively, 40, 33, 30 kDa apparent molecular weight, corresponding to P-CP43, P-D2 and P-D1, according to a previous report (Nath et al., 2013a). The intensity of the two lower MW bands was strongly increased in the HL vs D WT samples, whereas no such increase was detected in the *stn8* samples (Figure 2B). Immunoblot analysis using antibodies specific for CP29, D1 and D2 showed that P-CP29 co-migrated with P-D1 (Figure 2B). In order to verify whether the low MW anti-P-Thr-reactive band could be P-CP29 co-migrating with P-D1, we fractionated thylakoid proteins by sucrose-gradient ultracentrifugation upon solubilization with β-DDM, as previously reported (Betterle et al., 2015) and probed separately monomeric LHC proteins (including CP29) and PSII core components, which are characterized by a different sedimentation velocity in the gradient (Figure 3A). The polypeptides from the upper, Chl *b*-enriched, gradient band were depleted of PSII core components whereas the lower, Chl *a*-enriched gradient band contained PSII core complexes as shown by their reactivity to anti-D1 and anti-D2 antibodies while lacking CP29 (Figure 3B). When probing the two bands from the gradient with anti P-Thr antibody (Figure 3C), three bands were detected in the PSII core fraction with a migration pattern similar to that observed in HL-treated thylakoids. Thus, these bands corresponded to P-CP43, P-D1 and P-D2. Instead, the monomeric antenna fractions from HL samples contained a single P-Thr reactive band with a 30 kDa apparent molecular weight. No signal was detected in the dark adapted sample (Figure 3C). We conclude that P-CP29 and P-D1 co-migrated in SDS-PAGE from whole thylakoid samples clarifying why the HL-

dependent phosphorylation of CP29 remained undetected in previous work with the rice *stn8* mutant (Nath et al., 2013a).

The involvement of STN8 kinase in CP29 phosphorylation and the upper limit of 50% P-CP29 vs un-phosphorylated CP29 suggested a fraction of this protein is not accessible to the kinase. We thus proceeded to determine its distribution among thylakoid domains. To this aim, we isolated intact chloroplasts and thylakoids from dark adapted WT rice plants and, upon SDS-PAGE, detected STN8 by immunoblot analysis with anti-STN8 antibody. Thylakoids from *stn8* plants were also included as a control of the anti-STN8 reaction (Figure 4). The STN8 gene product was detected with the same intensity in chloroplasts and thylakoid samples consisting with STN8 being fully bound to the thylakoid membrane. A faint background signal was present in the sample loaded with thylakoids from *stn8* rice plants although with slightly higher apparent MW. To determine STN8 protein accumulation level, compared to STN7, in greenhouse grown plants, his-tagged STN8 and STN7 were expressed in *Escherichia coli* and recovered as inclusion bodies. Recombinant proteins were then purified by affinity chromatography in denaturating condition and the purity of the recombinant proteins was determined by SDS-PAGE (Figure 5A). Coomassie binding capacity of STN7 and STN8 was normalized based on the content in basic residues and aromatic residues (Syrovy and Hodny, 1991). Different amounts of the recombinant proteins were loaded together with rice wild-type thylakoids, and an immunoblot analysis was performed using antibodies specific for the two kinases (Figure 5B and C). The signal obtained from the recombinant proteins was used to generate a calibration line to determine the relative amount of the two kinases in thylakoid membranes based on the intensity of immunoblot signals yielded by the recombinant proteins and by the endogenous kinases

in thylakoid samples. We obtained a content of STN7 in thylakoids approximately 2 times higher with respect to STN8. An additional factor is the distribution of these enzymes among thylakoid domains: to assess thylakoid domain distribution of STN8 and STN7 kinases, we proceeded to solubilize stacked thylakoid membranes by using increasing concentrations of α-dodecylmaltoside (α-DDM), a treatment which preferentially solubilizes stroma-exposed thylakoid membranes and allows to harvest fractions of paired grana membrane discs of different diameter by differential centrifugation (Morosinotto et al., 2010). Consistent with previous reports (Pinnola et al., 2015), the Chl *a*/*b* ratio decreased in the pellet fraction by increasing the detergent concentration (Table 1). The SDS-PAGE analysis of these fractions showed that ATPase, which is a protein complex restricted to stroma membranes (Staehelin and Arntzen, 1983), could not be detected in the collected membranes upon treatment with high α-DDM concentrations (Figure 6) whereas LHCII polypeptides were enriched. The efficiency of the fractionation was further confirmed by the increased detection of CP43 and CP29 as markers for PSII, and depletion of LHCI, a marker for PSI. In particular, CP29 was progressively enriched in the pellet fractions whereas LHCI content declined. Detection of STN7 and STN8 kinases in these fractions using anti-STN7 and anti-STN8 antibodies (Figure 6) showed that STN7 was fully depleted from membranes collected upon treatment with α-DDM as in the case of ATPase. The pattern obtained with anti-STN8 antibody was, however, different since a detectable level of STN8 was present in the pellet even at higher α-DDM concentrations, a condition where the membranes are highly enriched in PSII core subunits and thus correspond to the inner region of grana partitions (Pinnola et al., 2015). Thylakoids from *stn8* plants were also included as a control of the anti-STN8 reaction. Thus, both STN7 and STN8 mainly partitioned in

stroma-exposed membranes, as evaluated by the ready solubilization of a large fraction of both antigens at low α-DDM concentrations. However, whereas further solubilization rapidly led to STN7 depletion, a significant fraction of STN8 was still detected in fractions corresponding to grana-partitions-derived regions. We calculated the content of STN8 in solubilized membranes by densitometry analysis of the corresponding western blot signal (Figure 6), normalized to the amount of STN8 present in the insolubilized thylakoids. We obtained a figure of 18% and 3% STN8 detectable in the membranes solubilized with the two highest α-DDM concentrations with respect to its content in whole thylakoids. STN7 was no longer detectable in these two solubilized fractions.

It was recently reported that STN7 and PPH1 are involved in phosphorylation of LHCII but not of CP29 in rice (Betterle et al., 2015; see also Figure 1). We thus proceeded to verify whether dephosphorylation of CP29 was catalyzed by PBCP phosphatase, responsible for PSII core components dephosphorylation in *Arabidopsis thaliana* (Samol et al., 2012). Since a knock-out line of the PBCP rice orthologue is not available, we proceeded to the preparation of recombinant *Os*PBCP. To this aim, we identified a putative sequence in rice (LOC_Os01g07090) based on the phylogenetic analysis of *At*PBCP orthologs (Samol et al., 2012). The mature coding sequence was amplified from cDNA *Oryza sativa* cv. Nipponbare, excluding the transit peptide sequence deduced through bioinformatic analysis with ChloroP. An N-terminally HIS-tagged PBCP (rPBCP) was expressed in *Escherichia coli*, followed by affinity chromatography in denaturing conditions. Upon verification of eluted fractions with SDS-PAGE (Figure 7A), fraction rPBCP-EL.3 was refolded *in vitro* according to a previously described protocol (Pribil et al., 2010). The phosphatase activity of rPBCP

was verified using para-nitrophenylphosphate (pNPP), as previously described (Samol et al., 2012). The specific activity was enhanced by the presence of DTT, Mn^{2+} cation and α-DDM (Figure 7B). On this basis we conclude that an active recombinant *Os*PBCP was successfully obtained.

We then proceeded to verify if rPBCP was active specifically on P-CP29. To this aim we used as substrate thylakoids isolated from HL-treated rice WT plants. The substrate was incubated for different time lengths either with or without rPBCP. After incubation, the reaction mixtures were precipitated in cold acetone and loaded on an SDS-PAGE followed by immunoblot analysis with the anti-CP29 antibody. rPBCP was effective in de-phosphorylating P-CP29 contained in thylakoids as judged from the complete depletion of the P-CP29 band upon 2 h treatment (Figure 7C) with a corresponding accumulation of CP29. Dephosphorylation activity was also detected in samples without addition of rPBCP, possibly due to the presence of the endogenous enzyme *Os*PBCP in traces. Also, we solubilized HL-treated thylakoids with α-DDM and purified monomeric antenna complexes, including P-CP29, by sucrose gradient ultracentrifugation. This monomeric LHC fraction was used as the substrate for rPBCP, whose activity was followed by using anti-PThr antibody, capable of recognizing P-CP29 (Figure 7D). Upon 2 hours treatment with rPBCP, P-CP29 was totally absent, while no de-phosphorylation activity was detected in the samples incubated without the recombinant enzyme. The specific activity of rPBCP against P-CP29 vs P-LHCII was also determined. WT rice leaves were treated with PSII-specific light for inducing LHCII phosphorylation (Pesaresi et al., 2009), and trimeric LHCII was purified by sucrose gradient centrifugation. Assay of P-LHCII with anti-PThr showed that 2 hours

incubation of P-LHCII with rPBCP yielded a very low decrease in P-LHCII (Figure 7E), if any. The P-LHCII signal was still evident upon overnight treatment although decreased. Thus, the activity of rPBCP on P-LHCII was far lower than on P-CP29.

DISCUSSION

High light-induced phosphorylation of CP29 was reported in monocots, including corn, rye, barley and rice (Bergantino et al., 1995; Mauro et al., 1997; Hwang et al., 2003; Pursiheimo et al., 2003; Liu et al., 2009; Chen et al., 2009, 2013); yet, the identity of the kinase is unknown. Despite *Os*CP29 (LHCB4) is a member of the LHC protein family, sharing high homology to LHCII including a similar structural organization (Liu et al., 2004; Pan et al., 2011), it is not phosphorylated by the STN7 kinase (Betterle et al., 2015), responsible for activity on LHCII (Bellafiore et al., 2005). This is surprising since CP29 and LHCII are near-neighbors within PSII supercomplexes (Bassi and Dainese, 1992; Boekema et al., 1999; Caffarri et al., 2009). The second-best candidate for the role of CP29 kinase is the STN8 kinase because it is the only known threonine kinase enzyme, besides STN7, associated to maize thylakoids (Friso et al., 2010). Moreover, differently from STN7, it is not inhibited by HL (Bonardi et al., 2005; Vainonen et al., 2005), a property consistent with CP29 phosphorylation occurring under excess light (Bergantino et al., 1995). Indeed, STN8 was shown to act on non-homologous and structurally unrelated substrates, i.e. the different PSII core proteins (Bonardi et al., 2005; Nath et al., 2013a).

The comparison of *Oryza sativa* WT and thylakoid kinase mutants, either dark adapted or HL treated, clearly showed that P-CP29 could not be detected in the absence of STN8 kinase. This result was evident upon analysis by SDS-PAGE followed by

immunoblot (Figure 1) or by Coomassie stain (Figure 2A). Due to the slower migration of rice P-CP29 with respect to CP29 in SDS-PAGE gels (Bergantino et al., 1998; Hwang et al., 2003; Liu et al., 2009; Chen et al., 2009), CP29 phosphorylation in rice was previously unrecognized (Nath et al., 2013a): in fact, P-CP29 co-migrates with heavily phosphorylated D1 and D2 subunits that form diffuse bands over the ~30 kDa MW range (Figure 2B). Here, we show that the combined use of a CP29 antibody and a high-resolution gel system allows for a clear detection of P-CP29 (Figure 2A). Moreover, a complete separation among P-PSII core subunits vs P-CP29 can be obtained by a pre-fractionation of the thylakoid membranes using sucrose gradient ultracentrifugation (Figure 3A). This technique allowed to separate solubilized complexes of different molecular weights, indeed PSII core is a complex of 300 kDa including P-CP43, P-D1 and P-D2, instead P-CP29 is a smaller monomeric holoprotein of ~40 kDa including chlorophyll and carotenoid co-factors (Figure 3A, B, C). We conclude that the long seeked CP29 kinase can be indeed identified with *Os*STN8.

CP29 is not the only substrate of *Os*STN8. Rather, this kinase retains the main substrates previously determined for the orthologue enzyme in *Arabidopsis thaliana*, namely CP43, D1 and D2 (Bonardi et al., 2005; Vainonen et al., 2005) as shown by the lack of P-CP43, P-D1 and P-D2 bands in immunoblots from HL-*stn8* thylakoids (Figure 2B). The inclusion of CP29 as a substrate for *Os*STN8 may depend on differences in the kinase or in the structure of CP29. *Os*CP29 is encoded by a single gene in rice (Loc_Os07g37240) whereas three different orthologues are present in *Arabidopsis thaliana* (LHCB4.1, AT5G01530; LHCB4.2, AT3G08940; LHCB4.3, AT2G37140). It was shown by sequence alignments that the three isoforms of *At*CP29 are highly conserved to the rice orthologue gene, and even the phosphorylation site of *Os*CP29,

threonine-82, is maintained (Betterle et al., 2015), suggesting changes in the kinase structure are responsible for the wider substrate specificity. This is supported by two evidences: (i) only traces of P-CP29 were found in Arabidopsis as a result of STN7 kinase activity (Tikkanen et al., 2006; Fristedt and Vener, 2011); (ii) *Os*STN8 and *At*STN8, although sharing high similarity in the C-terminal catalytic domain, are divergent in their N-termini, a domain suggested to be involved in substrate binding (Betterle et al., 2015).

A further substrate-related observation concerns the P-CP29/CP29 ratio that can be obtained with different durations of the HL treatment and was observed to tend to 1:1 without exceeding this value even upon long exposure. This seems a general feature in several species, including corn (Bergantino et al., 1995) that cannot be easily rationalized. One hypothesis is based on the dimeric structure of PSII supercomplexes (Santini et al., 1994; Boekema et al., 1999) and assumes that the kinase might have access to one monomer only. Verification of this hypothesis is difficult, since even the mildest solubilization of thylakoid membranes cannot preserve interactions between PSII and the STN7 or STN8 kinases (Lemeille et al., 2009). We observed that the level of CP29 phosphorylation in HL treated WT and overexpressing STN8 plants was equal (Figure 1), implying that the amount of STN8 kinase was not a limiting factor and suggesting the topological distribution of the kinase could be important. It should be considered that STN8 requires the reduction of cytochrome b_6f (cyt b_6f) for its activation: indeed, no phosphorylation of CP29 was observed in the presence of DCMU or DBMIB (Pursiheimo et al., 2003; Betterle et al., 2015), two compounds blocking electron transfer from PSII to cyt b_6f, suggesting a mandatory interaction between STN8 and reduced cyt b_6f. The latter was recently shown to be distributed not only in the

stroma lamellae but also in the grana domain (Johnson et al., 2014), where the STN8 substrate (CP29) is also present. The kinase access to 50% of CP29 could be explained by the localization of the kinase within a specific (sub)domain of grana partitions which hosts PSII supercomplexes including CP29 in contrast with non-CP29 PSII complexes that can be also found in stroma-exposed thylakoid domains and agranal chloroplasts. However, the fraction of CP29-depleted PSII complexes was shown to be very low (Santini et al., 1994). The thylakoid content of STN7 was 2 times higher than STN8 (Figure 5) and was excluded from grana stacks, showing a behavior similar to the ATPase. Instead, a significant fraction of STN8 was located in preparations of grana partitions in agreement with previous reports in *Arabidopsis thaliana* (Wunder et al., 2013) (Figure 6). This different distribution was evident even upon treatment with the highest detergent strength which trims away grana margins (Morosinotto et al., 2010; Pinnola et al., 2015). Thus, the presence of STN8 in grana domains, different from STN7, is consistent with the presence of both its activator and its substrate. Although present in grana membranes, *Os*STN8 is mostly removed by DDM final concentrations of 0.75%, implying the center of grana partitions was strongly depleted of both kinases (Figure 6). Indeed, the mobility of PSII complexes within grana membranes appears to be limited (Kirchoff, 2014). We suggest that the major reason for limitation of P-CP29 phosphorylation level to 50% is the lack of *Os*STN8 from the center of grana disks.

As for the difference between monocots and dicots, the finding that *Os*STN8 was localized in similar thylakoid domains as *At*STN8, supports the conclusion that phosphorylation of CP29 in rice is allowed by some critical differences in the sequences of Arabidopsis and rice STN8, as previously suggested (Betterle et al., 2015), rather than by their differential localization in thylakoid membranes.

Phosphorylation events are regulated by antagonistic kinase/phosphatase pairs, thus we attempted identification of the phosphatase acting on P-CP29. PPH1 phosphatase was recently shown not to be involved in the process of CP29 dephosphorylation (Betterle et al., 2015). We verified the hypothesis that the enzyme antagonist to STN8 activity for PSII core phosphorylation, namely PBCP in *Arabidopsis thaliana* (Samol et al., 2012), was involved. The orthologue maize gene product (gene model GRMZM2G003096_P01) was found to be intrinsic of thylakoid membranes, as for maize PPH1 orthologue (Friso et al., 2010). Since no additional phosphatases were detected by proteomic analysis (Friso et al., 2010), the hypothesis appeared to be well grounded. The orthologue sequence from rice expressed in *Escherichia coli* (Figure 7A) showed an activity strictly dependent on the presence of both Mn^{2+} cation and DTT which could be significantly increased in the presence of α-DDM (Figure 7B), in agreement with previous reports with *At*PBCP (Samol et al., 2012). We observed that recombinant *Os*PBCP (rPBCP) was highly active and capable of fully dephosphorylating P-CP29 in thylakoid membranes isolated from HL-treated WT rice leaves (Figure 7C). De-phosphorylation activity of rPBCP on P-CP29 was far higher than on P-LHCII (Figure 7D, E) suggesting P-CP29 rather than P-LHCII is its physiological substrate. These results further supported the assessment that *Os*PBCP protein is not only responsible for PSII core subunit dephosphorylation, as previously observed in *Arabidopsis thaliana* (Samol et al., 2012), but it is also responsible for P-CP29 dephosphorylation in monocots. Although the absence of a rice *pbcp* mutant does not allow to prove that beyond any doubt, this conclusion is consistent with the observation that the phosphorylation of substrates in thylakoids is regulated by a specific kinase and phosphatase pair, as observed for the regulation of a) P-LHCII,

involving STN7 and PPH1/TAP38 (Rochaix et al., 2012); b), the phosphorylation of PSII core subunits, involving STN8 and PBCP (Nath et al., 2013b).

In conclusion, CP29 phosphorylation is involved in photoprotection (Betterle et al., 2015; Mauro et al., 1997) and caused a protein conformational change (Croce et al., 1996). Based on our data, we hypothesize that the activity of STN8 kinase is necessary for the repair of photo-damaged PSII though phosphorylation of D1 subunit of PSII, a step essential for the PSII repair cycle (Pesaresi et al., 2011; Tikkanen and Aro, 2012; Nath et al., 2013b). In addition, the phosphorylation of CP29, which occurs in the same photoinhibitory conditions, ensures the maintenance of a quenched state of the PSII antenna system (Mauro et al., 1997). This is useful to minimize ROS production during the disassembling and repair of damaged PSII. In order to confirm this hypothesis for the function of CP29 phosphorylation, the analysis of a mutant depleted of CP29 phosphorylation site will be of the utmost importance.

MATERIAL AND METHODS

Plant materials and growth condition - Oryza sativa stn8 mutant was obtained from a T-DNA insertional mutant library (Nath et al., 2013), whereas *stn7* and *pph1* mutants were respectively obtained from *Oryza Tag Line* and RMD rice mutant libraries (Betterle et al., 2015). Plants were grown in greenhouse at 35/28°C (day/night) with natural light during warm seasons. Leaves detached from rice plants were dark adapted for two hours and then treated with high light (1500 μmol photons m^{-2} s^{-1}) using halogen lamps filtered by a 1-cm water layer to remove infrared radiation. To induce LHCII phosphorylation, dark-adapted leaves were treated for one hour with orange light at 100

μmol photons m^{-2} s^{-1} (30 W warm white fluorescent lamps filtered through Lee Filters 105 Orange) as previously reported (Pesaresi et al., 2009).

Membrane isolation - Functional chloroplasts/thylakoids were isolated as previously described (Casazza et al., 2001). Grana membranes were prepared according to previous report (Morosinotto et al., 2010).

Sucrose gradient fractionation - Membranes corresponding to 150 mg of chlorophyll were washed with 5 mM EDTA and then solubilized with 0.6% β-DDM (Figure 3) or α-DDM (Figure 7). Solubilized samples were then fractionated by ultracentrifugation in a 0.1‑1 M sucrose gradient containing 0.03% of the detergent used for thylakoid solubilization and 20 mM HEPES, pH 7.5 (SW60 Ti Rotor, 5h 30min at 60,000 rpm, 4 °C).

Pigment Composition – Chlorophyll *a*/*b* ratio of isolated membranes was analyzed by fitting the spectrum of the 80% acetone-extracted pigments with the spectra of the individual pigments in acetone as described previously (Croce et al., 2002).

Gel Electrophoresis and Immunoblotting - SDS-PAGE analysis was performed with the Tris-Glycine buffer system (Laemmli, 1970), with the addition of 2 M urea to the running gel to separate phosphorylated and unphosphorylated CP29 polypeptides. For western blot analysis, chloroplast or thylakoid samples were loaded on SDS-PAGE and electroblotted on nitrocellulose membranes or PVDF membranes (0.45 μm pore size, Millipore). Proteins were detected using homemade anti-CP29, anti-CP43, anti-LHCA, anti-Rubisco, anti-STN7 or anti-STN8 antisera, all raised in rabbit, and an alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich). In the cases of anti-STN7 and anti-STN8 antisera, recombinant *Arabidopsis thaliana* mature STN7 and STN8 were provided by prof. Jean David Rochaix, University of Geneva) and used for rabbit

immunization. For immunoblotting analysis using anti-CP29 antibody (Agrisera), thylakoid samples were isolated as previously described (Oh et al., 2009). For detection of phosphoproteins, anti-phosphothreonine polyclonal antibody (Cell Signaling) was used. Signal amplitude was quantified using GelPro 3.1 software (Bio-Rad).

Expression of recombinant proteins – *OsPbcp* gene sequence was identified upon alignment of *AtPbcp* gene sequence (Samol et al., 2012) with rice genome. Chloroplast transition peptides of *Stn7* (LOC_Os05g47560), *Stn8* (LOC_Os05g40180) and *Pbcp* (LOC_Os01g07090) gene products were determined using ChloroP method. cDNA sequences of the predicted mature proteins were cloned into pDEST17 (Invitrogen), a Gateway compatible vector to allow for N-terminal 6x-His tagging, and expressed in *Escherichia coli* strain BL21. Recombinant proteins were purified using a protocol described previously (Paulsen et al., 1993) and resuspended in denaturant solution (Tris 20 mM pH 7.4, NaCl 300 mM, Urea 6 M). Denatured proteins were loaded in Ni-Sepharose column and elution of tagged proteins was performed in a solution containing Tris 20 mM pH 7.4, SDS 0.2 % and 200 mM Imidazole. Denatured rPBCP was reconstituted as recently described (Pribil et al., 2010). Subsequently, 1 µg of phosphatase was incubated together with thylakoid membranes corresponding to 1 µg of total chlorophylls. The dephosphorylation reaction was performed in 100 µl containing 0.03% (w/v) α-DDM, NaCl 100mM, 10 mM $MnCl_2$, 10 mM DTT, 20 mM HEPES (pH 7.5), at 25 °C for 2 h and overnight. The reaction mixture was precipitated in cold acetone and then loaded on a SDS-PAGE. Immunoblot was finally performed using an anti-CP29 or anti-PThr specific antibodies, as described above.

ACKNOWLEDGEMENTS

Research performed at the University of Verona was supported by ITN Accliphot and ITN S2B. Research performed at the Pusan National University the Basic Science Research Program of the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT, and Future Planning (Grant No. NRF-2014R1A2A2A01005741); as well as by the "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01117101)", Rural Development Administration, Republic of Korea. The authors declare no conflict of interest.

REFERENCES

Allen, K.D. and Staehelin, L.A. (1992). Biochemical-Characterization of Photosystem-II Antenna Polypeptides in Grana and Stroma Membranes of Spinach. *Plant Physiol.* **100**:1517–1526.

Aro, E., Mccaffery, S., and Anderson, J.M. (1994). Recovery from Photoinhibition in Peas (*Pisum sativum* L.) Acclimated to Varying Growth Irradiances . *Plant Physiol.* **104**:1033–1041.

Bassi, R., Giacometti, G.M., and Simpson, D. (1988). Changes in the composition of stroma lamellae following state I-state II transitions. *Biochim. Biophys. Acta* **935:**152–165**.**

Bassi, R. and Dainese, P. (1992). A supramolecular light-harvesting complex from chloroplast photosystem-II membranes. *Eur. J. Biochem.* **204**:317–326.

Bassi, R., Marquardt, J., and Lavergne, J. (1995). Biochemical and functional properties of photosystem II in agranal membranes from maize mesophyll and

bundle sheath chloroplasts. *Eur. J. Biochem.* **233**:709–719.

Bellafiore, S., Barneche, F., Peltier, G., and Rochaix, J.-D. (2005). State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. *Nature* **433**:892–895.

Bergantino, E., Dainese, P., Cerovic, Z., Sechi, S., and Bassi, R. (1995). A post-translational modification of the photosystem II subinut CP29 protects maize from cold stress. *J. Biol. Chem.* **270**:8474–8481.

Bergantino, E., Sandonà, D., Cugini, D., and Bassi, R. (1998). The photosystem II subunit CP29 can be phosphorylated in both C3 and C4 plants as suggested by sequence analysis. *Plant Mol. Biol.* **36**:11–22.

Betterle, N., Ballottari, M., Baginsky, S., and Bassi, R. (2015). High Light-Dependent Phosphorylation of Photosystem II Inner Antenna CP29 in Monocots Is STN7 Independent and Enhances Nonphotochemical Quenching. *Plant Physiol.* **167**:457–471.

Boekema, E., van Roon, H., and van Breemen, J. (1999). Supramolecular organization of photosystem II and its light-harvesting in partially solubilized photosystem II membranes. *Eur. J. Biochem.* **452**:444–452.

Bonardi, V., Pesaresi, P., Becker, T., Schleiff, E., Wagner, R., Pfannschmidt, T., Jahns, P., and Leister, D. (2005). Photosystem II core phosphorylation and photosynthetic acclimation require two different protein kinases. *Nature* **437**:1179–1182.

Caffarri, S., Kouril, R., Kereïche, S., Boekema, E.J., and Croce, R. (2009). Functional architecture of higher plant photosystem II supercomplexes. *EMBO J.* **28**:3052–63.

Casazza, P., Tarantino, D., and Soave, C. (2001). Preparation and functional characterization of thylakoids from Arabidopsis thaliana. *Photosynth. Res.* **68**:175–180.

Chen, Y.-E., Yuan, S., Du, J.-B., Xu, M.-Y., Zhang, Z.-W., and Lin, H.-H. (2009). Phosphorylation of photosynthetic antenna protein CP29 and photosystem II structure changes in monocotyledonous plants under environmental stresses. *Biochemistry* **48**:9757–9763.

Chen, Y.-E., Zhao, Z.-Y., Zhang, H.-Y., Zeng, X.-Y., and Yuan, S. (2013). The significance of CP29 reversible phosphorylation in thylakoids of higher plants under environmental stresses. *J. Exp. Bot.* **64**:1167–1178.

Crepin, A. and Caffarri, S. (2015). The specific localizations of phosphorylated Lhcb1 and Lhcb2 isoforms reveal the role of Lhcb2 in the formation of the PSI-LHCII supercomplex in Arabidopsis during state transitions. *Biochim. Biophys. Acta - Bioenerg.* **1847**:1539–1548.

Croce, R., Breton, J., and Bassi, R. (1996). Conformational changes induced by phosphorylation in the CP29 subunit of photosystem II. *Biochemistry* **35**:11142–11148.

Croce, R., Canino, G., Ros, F., and Bassi, R. (2002). Chromophore organization in the higher-plant photosystem II antenna protein CP26. *Biochemistry* **41**:7334–7343.

de Bianchi, S., Ballottari, M., Dall'osto, L., and Bassi, R. (2010). Regulation of plant light harvesting by thermal dissipation of excess energy. *Biochem. Soc. Trans.* **38**:651–660.

Friso, G., Majeran, W., Huang, M., Sun, Q., and van Wijk, K.J. (2010). Reconstruction of metabolic pathways, protein expression, and homeostasis

machineries across maize bundle sheath and mesophyll chloroplasts: large-scale quantitative proteomics using the first maize genome assembly. *Plant Physiol.* **152**:1219–1250.

Fristedt, R. and Vener, A. V (2011). High light induced disassembly of photosystem II supercomplexes in Arabidopsis requires STN7-dependent phosphorylation of CP29. *PLoS One* **6**: e24565.

Galka, P., Santabarbara, S., Khuong, T.T.H., Degand, H., Morsomme, P., Jennings, R.C., Boekema, E.J., and Caffarri, S. (2012). Functional analyses of the plant photosystem I-light-harvesting complex II supercomplex reveal that light-harvesting complex II loosely bound to photosystem II is a very efficient antenna for photosystem I in state II. *Plant Cell* **24**:2963–2978.

Horton, P., Ruban, a. V., and Walters, R.G. (1996). Regulation of Light Harvesting in Green Plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**:655–684.

Hwang, H., Xu, C., Moon, B., and Lee, C. (2003). Recovery from low-temperature photoinhibition is related to dephosphorylation of phosphorylated CP29 rather than zeaxanthin epoxidation in rice leaves. *J. Plant Biol.* **46**:122–129.

Johnson, M.P., Vasilev, C., Olsen, J.D., and Hunter, C.N. (2014). Nanodomains of Cytochrome b_6f and Photosystem II Complexes in Spinach Grana Thylakoid Membranes. *Plant Cell* **26**: 3051–3061.

Kirchhoff, H. (2014) Diffusion of molecules and macromolecules in thylakoid membranes. *Biochim. Biophys. Acta* **1837**: 495-502

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.

Lemeille, S., Willig, A., Depège-Fargeix, N., Delessert, C., Bassi, R., and Rochaix,

J.-D. (2009). Analysis of the chloroplast protein kinase Stt7 during state transitions. *PLoS Biol.* **7**: e45.

Liu, W.-J., Chen, Y.-E., Tian, W.-J., Du, J.-B., Zhang, Z.-W., Xu, F., Zhang, F., Yuan, S., and Lin, H.-H. (2009). Dephosphorylation of photosystem II proteins and phosphorylation of CP29 in barley photosynthetic membranes as a response to water stress. *Biochim. Biophys. Acta* **1787**:1238–1245.

Liu, Z., Yan, H., Wang, K., Kuang, T., Zhang, J., Gui, L., An, X., and Chang, W. (2004). Crystal structure of spinach major light-harvesting complex at 2.72 A resolution. *Nature* **428**:287–292.

Longoni, P., Douchi, D., Cariti, F., Fucile, G., and Goldschmidt-Clermont, M. (2015). Phosphorylation of the Lhcb2 isoform of Light Harvesting Complex II is central to state transitions. *Plant Physiol.* **169**: pp.01498.2015.

Mauro, S., Dainese, P., Lannoye, R., and Bassi, R. (1997). Cold-Resistant and Cold-Sensitive Maize Lines Differ in the Phosphorylation of the Photosystem II Subunit, CP29. *Plant Physiol.* **115**:171–180.

Morosinotto, T., Segalla, A., Giacometti, G.M., and Bassi, R. (2010). Purification of structurally intact grana from plants thylakoids membranes. *J. Bioenerg. Biomembr.* **42**:37–45.

Nath, K. et al. (2013a). Loss-of-function of OsSTN8 suppresses the photosystem II core protein phosphorylation and interferes with the photosystem II repair mechanism in rice (*Oryza sativa*). *Plant J.* **76**:675–686.

Nath, K., Jajoo, A., Poudyal, R.S., Timilsina, R., Park, Y.S., Aro, E.M., Nam, H.G., and Lee, C.H. (2013b). Towards a critical understanding of the photosystem II repair mechanism and its regulation during stress conditions. *FEBS Lett.*

587:3372–3381.

Navabpour, S., Morris, K., Allen, R., Harrison, E., A-H-Mackerness, S., and Buchanan-Wollaston, V. (2003). Expression of senescence-enhanced genes in response to oxidative stress. *J. Exp. Bot.* **54**:2285–2292.

Nelson, N. and Ben-Shem, A. (2004). The complex architecture of oxygenic photosynthesis. *Nat. Rev. Mol. Cell Biol.* **5**:971–982.

Niyogi, K.K. and Truong, T.B. (2013). Evolution of flexible non-photochemical quenching mechanisms that regulate light harvesting in oxygenic photosynthesis. *Curr. Opin. Plant Biol.* **16**:307–314.

Oh, M.-H., Safarova, R.B., Eu, Y.-J., Zulfugarov, I.S., Kim, J.-H., Hwang, H.J., Lee, C.B. and Lee, C.-H. (2009) Loss of peripheral polypeptides in the stromal side of photosystem I by light-chilling in cucumber leaves. *Photochem. Photobiol. Sci.* **8**:535-541

Pan, X., Li, M., Wan, T., Wang, L., Jia, C., Hou, Z., Zhao, X., Zhang, J., and Chang, W. (2011). Structural insights into energy regulation of light-harvesting complex CP29 from spinach. *Nat. Struct. Mol. Biol.* **18**:309–315.

Paulsen, H., Finkenzeller, B., and Kühlein, N. (1993). Pigments induce folding of light-harvesting chlorophyll a/b-binding protein. *Eur. J. Biochem.* **215**:809–816.

Pesaresi, P., Hertle, A., Pribil, M., Kleine, T., Wagner, R., Strissel, H., Ihnatowicz, A., Bonardi, V., Scharfenberg, M., Schneider, A., Pfannschmidt, T., and Leister, D. (2009). Arabidopsis STN7 kinase provides a link between short- and long-term photosynthetic acclimation. *Plant Cell* **21**:2402–2423

Pesaresi, P., Pribil, M., Wunder, T., and Leister, D. (2011). Dynamics of reversible protein phosphorylation in thylakoids of flowering plants: The roles of STN7,

STN8 and TAP38. *Biochim. Biophys. Acta - Bioenerg.* **1807**:887–896.

Pietrzykowska, M., Suorsa, M., Semchonok, D. a., Tikkanen, M., Boekema, E.J., Aro, E.-M.E.-M., and Jansson, S. (2014). The Light-Harvesting Chlorophyll a/b Binding Proteins Lhcb1 and Lhcb2 Play Complementary Roles during State Transitions in Arabidopsis. *Plant Cell* **26**:3646–3660.

Pinnola, A., Cazzaniga, S., Alboresi, A., Nevo, R., Levin-Zaidman, S., Reich, Z., and Bassi, R. (2015). Light-Harvesting Complex Stress-Related Proteins Catalyze Excess Energy Dissipation in Both Photosystems of Physcomitrella patens. *Plant Cell* **27**:3213–3227.

Pribil, M., Pesaresi, P., Hertle, A., Barbato, R., and Leister, D. (2010). Role of plastid protein phosphatase TAP38 in LHCII dephosphorylation and thylakoid electron flow. *PLoS Biol.* **8**: e1000288.

Pursiheimo, S., Martinsuo, P., Rintamäki, E., and Aro, E. (2003). Photosystem II protein phosphorylation follows four distinctly different regulatory patterns induced by environmental cues. *Plant Cell Environ* 26: 1995–2003

Rintamaki, E., Salonen, M., Suoranta, U.-M., Carlberg, I., Andersson, B., and Aro, E.-M. (1997). Phosphorylation of Light-harvesting Complex II and Photosystem II Core Proteins Shows Different Irradiance-dependent Regulation in Vivo: APPLICATION OF PHOSPHOTHREONINE ANTIBODIES TO ANALYSIS OF THYLAKOID PHOSPHOPROTEINS. *J. Biol. Chem.* **272**:30476–30482.

Rochaix, J.-D., Lemeille, S., Shapiguzov, A., Samol, I., Fucile, G., Willig, A., and Goldschmidt-Clermont, M. (2012). Protein kinases and phosphatases involved in the acclimation of the photosynthetic apparatus to a changing light environment. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **367**:3466–3474.

Samol, I., Shapiguzov, a, Ingelsson, B., Fucile, G., Crevecoeur, M., Vener, a V, Rochaix, J.D., and Goldschmidt-Clermont, M. (2012). Identification of a Photosystem II Phosphatase Involved in Light Acclimation in Arabidopsis. *Plant Cell* **24**:2596–2609.

Santini, C., Tidu, V., Tognon, G., Ghiretti Magaldi, a, and Bassi, R. (1994). Three-dimensional structure of the higher-plant photosystem II reaction centre and evidence for its dimeric organization in vivo. *Eur. J. Biochem.* **221**:307–315.

Shapiguzov, A., Chai, X., Fucile, G., Longoni, P., Zhang, L., and Rochaix, J.-D. (2016). Activation of the Stt7/STN7 kinase through dynamic interactions with the cytochrome b6f complex. *Plant Physiol.* **171**: pp.01893.2015.

Shapiguzov, A., Ingelsson, B., Samol, I., Andres, C., Kessler, F., Rochaix, J.-D., Vener, A. V, and Goldschmidt-Clermont, M. (2010). The PPH1 phosphatase is specifically involved in LHCII dephosphorylation and state transitions in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* **107**:4782–4787.

Staehelin, L.A. and Arntzen, C.J. (1983). Regulation of chloroplast membrane function: Protein phosphorilation changes the spatial organization of membranes components. *J. Cell Biol.* **97**:1327–1337.

Syrovy, L. and Hodny, Z. (1991). Staining and quantification of proteins separated by polyacrylamide gel electrophoresis. *J. Chromatogr.* **569**:175-196.

Tikkanen, M., Piippo, M., Suorsa, M., Sirpiö, S., Mulo, P., Vainonen, J., Vener, AV, Allahverdiyeva, Y. and Aro, E.M. (2006). State transitions revisited-a buffering system for dynamic low light acclimation of Arabidopsis. *Plant Mol. Biol.* **62**:779–793.

Tikkanen, M. and Aro, E.M. (2012). Thylakoid protein phosphorylation in dynamic

regulation of photosystem II in higher plants. *Biochim. Biophys. Acta - Bioenerg.* **1817**:232–238.

Vainonen, J.P., Hansson, M., and Vener, A. V (2005). STN8 protein kinase in Arabidopsis thaliana is specific in phosphorylation of photosystem II core proteins. *J. Biol. Chem.* **280**:33679–33686.

Wunder, T., Xu, W., Liu, Q., Wanner, G., Leister, D., and Pribil, M. (2013). The major thylakoid protein kinases STN7 and STN8 revisited: effects of altered STN8 levels and regulatory specificities of the STN kinases. *Front. Plant Sci.* **4**:417.

TABLE

Table 1. Determination of Chlorophyll *a/b* ratio of isolated membranes. Thylakoids from WT plants (WT-Thyl) and pellet fractions were obtained with differential solubilization, as previously described (Morosinotto et al., 2010; Pinnola et al, 2015). The spectrum of the 80% acetone-extracted pigments was analyzed by fitting the spectra of the individual pigments in acetone as previously described (Croce et al., 2002). *stn8* thylakoids were used as control.

	WT-Thyl	**WT-pellet 0.09%**	**WT-pellet 0.18%**	**WT-pellet 0.37%**	**WT-pellet 0.75%**	**stn8-Thyl**
Chl *a/b*	3.4	4.1	3.8	3.2	2.8	3.4

FIGURE LEGENDS

Figure 1: The STN8 protein kinase of rice is required for CP29 phosphorylation. Immunoblot analysis of thylakoids isolated from *Oryza sativa* wild-type (Hw, cv. Hwayoung; Np, cv. Nipponbare; Zh, cv. Zhonghua 11) and mutant plants recently described (Nath et al., 2013; Betterle et al., 2015). Before thylakoid isolation, leaves were dark-adapted for 12 hours (D) and then treated with white light (HL; 1500 μmol photons m^{-2} s^{-1}) for 60 minutes. Tris-Gly SDS-PAGE 10% acrylamide plus Urea 2M; Immunoblot was performed using anti-CP29 polyclonal antibody (Agrisera). 2 micrograms of total chlorophylls (Chl) per lane.

Figure 2: The STN8 protein kinase is responsible for both PSII core subunits and CP29 phosphorylation. A, SDS-PAGE and immunoblot analyses of thylakoids isolated from wild-type and *stn8 Oryza sativa* plants. Before thylakoids isolation, wild-type and *stn8* mutant leaves were dark-adapted for 2 hours (dark) and then treated with white light (HL; 1500 μmol photons m^{-2} s^{-1}) for 30 minutes. Tris-Gly SDS-PAGE 12-18% acrylamide plus Urea 3M; Immunoblot was performed using anti-CP29 polyclonal antibody. 3 micrograms of total chlorophylls (Chl) per lane. **B,** Analysis of thylakoid phosphoproteins using anti phospho-threonine antibody (Cell Signaling). Before thylakoids isolation, wild type and *stn8* mutant rice leaves were treated as previously described in A. A detail of Red Ponceau stained filter before immunoblot analysis, corresponding to the LHCII migration region, is shown as loading control. Antibodies against CP29, D1 and D2 were used for identify the electrophoretic migration of CP29, D1 and D2 proteins from thylakoids of HL treated WT leaves.

Figure 3: Demonstration of CP29 phosphorylation detection by anti phospho-threonine antibody. A, Sucrose gradient fractionation of mildly solubilized thylakoids and absorption spectra in the visible region. Thylakoids were isolated from WT rice leaves dark adapted or treated with white light (HL; 1500 µmol photons m^{-2} s^{-1}) for 30 minutes and then monomeric antenna proteins (mLHC) and PSII core (PSIIc) fractions were purified upon sucrose gradient ultracentrifugation of β-dodecylmaltoside solubilized thylakoids (Thyl). **B,** Immunoblot analysis of thylakoids and sucrose gradient isolated fractions. Antibodies against CP29, D1 and D2 were used. 0.75 micrograms of total chlorophylls (Chl) per lane with thylakoids samples, 0.25 micrograms of total chlorophylls (Chl) per lane with mLHC and PSIIc fractions. Tris-Gly SDS-PAGE 12% acrylamide plus Urea 3M. **C,** Analysis of thylakoid phosphoproteins using anti-phospho-threonine antibody (Cell Signaling). Samples used were collected from solubilized thylakoids from dark adapted leaves or treated with HL as shown in A.

Figure 4: Subcellular localization of STN8 kinase in rice. Determination of STN8 localization by immunoblot analysis on isolated chloroplasts or thylakoids. Chloroplasts and thylakoids were isolated from dark adapted WT rice leaves. Immunoblot analyses have been performed using anti-Rubisco, anti-STN8 and anti-CP29 antibodies. The presence of Rubisco reflected the presence of stromal components, while CP29 was a marker for thylakoid complexes. Thylakoids from *stn8* plants were used as a control for reliable STN8 detection, because of the presence of an aspecific band with similar molecular weight. One microgram of Chls per lane.

Figure 5: Determination of STN7 and STN8 kinases relative amount in isolated thylakoids. His-tagged proteins were purified by affinity chromatography and checked in Tris-Glycine SDS-PAGE. Immunoblot analyses were performed using anti-STN7 and anti-STN8 antibodies on these fractions and on thylakoid (WT-Thyl) samples isolated from wild-type plants. 0.75, 1.5 and 3 micrograms of Chls per lane were respectively loaded for WT Thyl, WT Thyl – 2X and WT Thyl – 4X. Quantification analysis was performed with a second independent preparation of thylakoids giving similar result.

Figure 6: Distribution of STN8 kinase in thylakoid membranes. Fractionation of WT thylakoid membranes as previously reported (Morosinotto et al., 2010) through solubilization with increasing amounts of α-dodecylmaltoside (Pellet-X%, where X% represents the final concentration of the detergent), followed by centrifugation for separating solubilized membranes from unsolubilized ones. The obtained fractions were used for the determination of STN8 and STN7 kinase localization in thylakoid membranes, using the analyses with antibodies against CP43, CP29 and LHCA as indicators for grana or stroma exposed membranes enrichments. Immunoblot analyses was performed using anti-STN7 and anti-STN8 antibodies. 1.5 micrograms of Chls per lane. Fractionation analysis was repeated giving similar result.

Figure 7: Recombinant PBCP of rice is able to dephosphorylate P-CP29. A, SDS-Page analysis of fractions resulted from affinity chromatography for purification of recombinant PBCP-HisTag in denaturant condition. 1 microgram of bovin serum albumin was loaded as a reference. **B,** *In vitro* spectrophotometric assay of recombinant

PBCP activity upon *in vitro* refolding. The dephosphorylation of pNPP was measured by the increase in absorption at 405 nm in 1-mL reaction volumes containing 1 microgram recombinant PBCP, 300 mM NaCl, 50 mM HEPES, pH 8.0, 10 mM pNPP, and the additives indicated. Error bars represent the SD of triplicate measurements. **C,** Thylakoid membranes were collected from high-light treated leaves of wild-type plants. Samples (1 microgram of Chls) was finally incubated for 2 hours/ovenight in the presence/absence of purified recombinant PBCP (1 microgram). Immunoblot analysis was performed using anti-CP29 antibody. **D**, Monomeric antenna complexes were purified by sucrose gradient fractionation of solubilized thylakoids used in C. Samples (0.25 micrograms of Chls) was treated as above. Immunoblot analysis was performed using anti-phosphothreonine antibody. **E,** Trimeric LHCII complexes were purified as in D, using thylakoids isolated from leaves treated with PSII-enriched light. Samples (0.25 micrograms of Chls) was treated as above. Immunoblot analysis was performed using anti-phosphothreonine antibody.

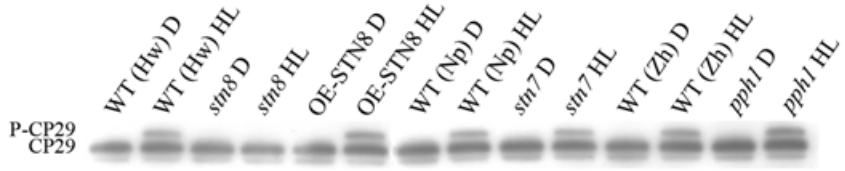

Figure 1: The STN8 protein kinase of rice is required for CP29 phosphorylation. Immunoblot analysis of thylakoids isolated from *Oryza sativa* wild-type (Hw, cv. Hwayoung; Np, cv. Nipponbare; Zh, cv. Zhonghua 11) and mutant plants recently described (Nath et al., 2013; Betterle et al., 2015). Before thylakoid isolation, leaves were dark-adapted for 12 hours (D) and then treated with white light (HL; 1500 µmol photons m–2 s–1) for 60 minutes. Tris-Gly SDS-PAGE 10% acrylamide plus Urea 2M; Immunoblot was performed using anti-CP29 polyclonal antibody (Agrisera). 2 µg of total chlorophylls (Chl) per lane.

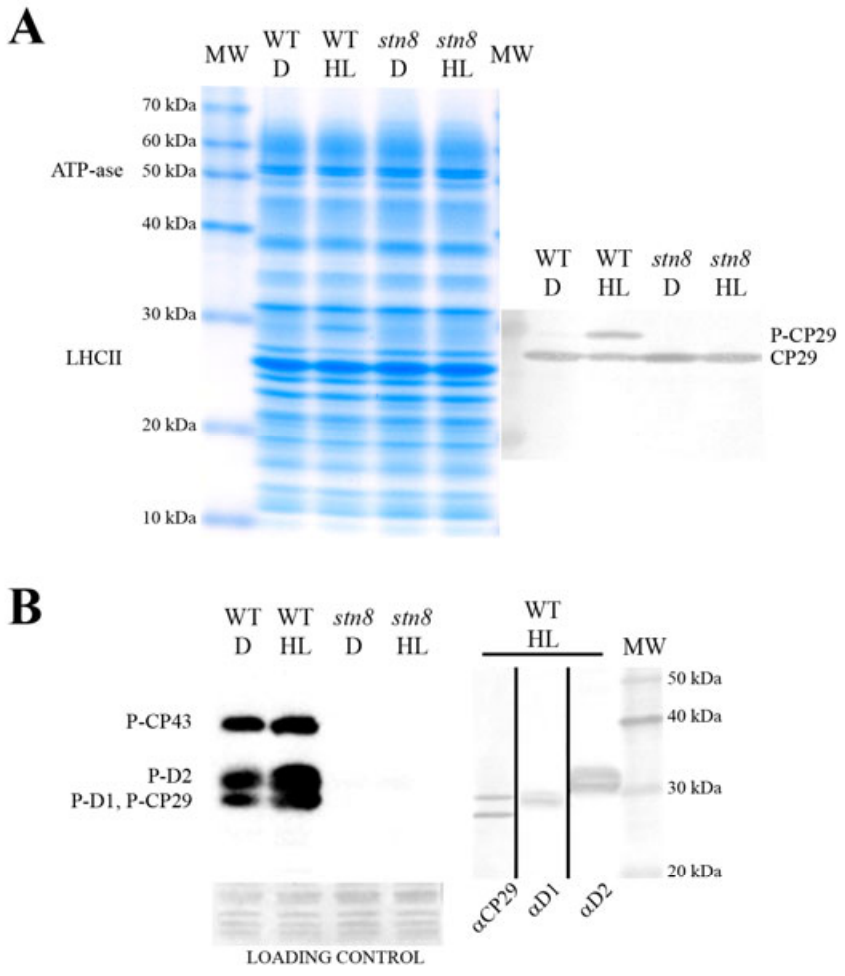

Figure 2: The STN8 protein kinase is responsible for both PSII core subunits and CP29 phosphorylation. A, SDS-PAGE and immunoblot analyses of thylakoids isolated from wild-type and *stn8 Oryza sativa* plants. Before thylakoids isolation, wild-type and *stn8* mutant leaves were dark-adapted for 2 hours (dark) and then treated with white light (HL; 1500 µmol photons m–2 s–1) for 30 minutes. Tris-Gly SDS-PAGE 12-18% acrylamide plus Urea 3M; Immunoblot was performed using anti-CP29 polyclonal antibody. 3 micrograms of total chlorophylls (Chl) per lane. **B,** Analysis of thylakoid phosphoproteins using anti phospho-threonine antibody (Cell Signaling). Before thylakoids isolation, wild type and *stn8* mutant rice leaves were treated as previously described in A. A detail of Red Ponceau stained filter before immunoblot analysis, corresponding to the LHCII migration region, is shown as loading control. Antibodies against CP29, D1 and D2 were used for identify the electrophoretic migration of CP29, D1 and D2 proteins from thylakoids of HL treated WT leaves.

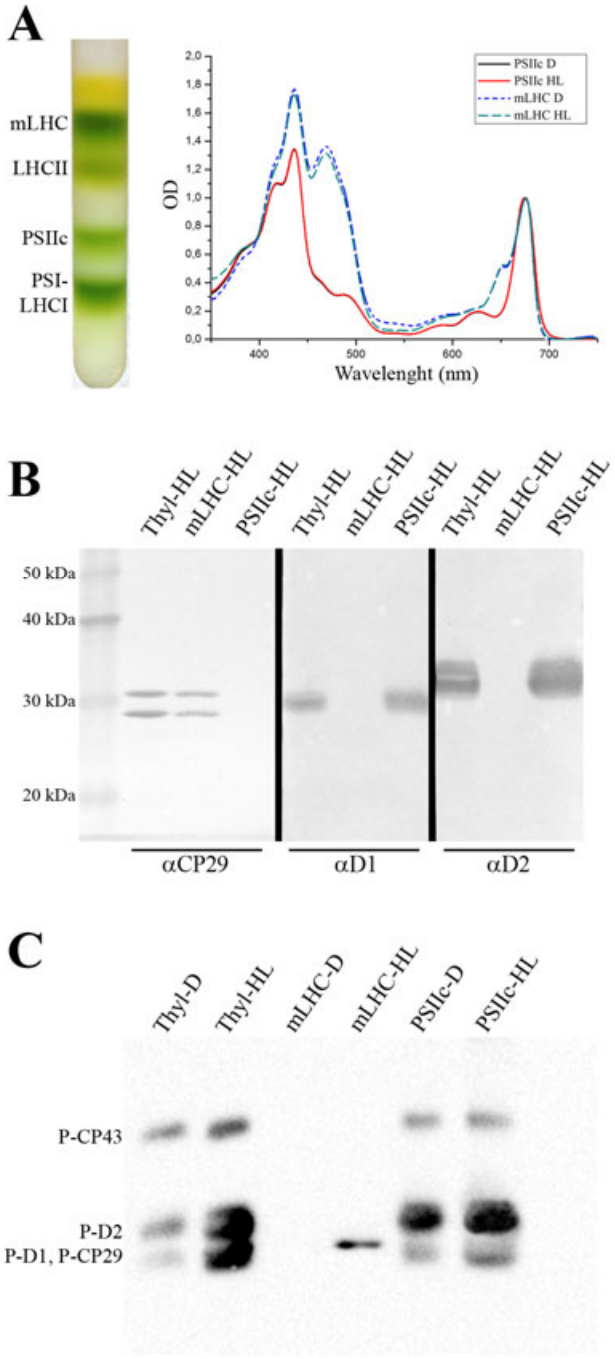

Figure 3: Demonstration of CP29 phosphorylation detection by anti phospho-threonine antibody. A, Sucrose gradient fractionation of mildly solubilized thylakoids and absorption spectra in the visible region. Thylakoids were isolated from WT rice leaves dark adapted or treated with white light (HL; 1500 µmol photons m–2 s–1) for 30 minutes and then monomeric antenna proteins (mLHC) and PSII core (PSIIc) fractions were purified upon sucrose gradient ultracentrifugation of β-dodecylmaltoside solubilized thylakoids (Thyl). **B,** Immunoblot analysis of thylakoids and sucrose gradient isolated fractions. Antibodies against CP29, D1 and D2 were used. 0.75 micrograms of total chlorophylls (Chl) per lane with thylakoids samples, 0.25 micrograms of total chlorophylls (Chl) per lane with mLHC and PSIIc fractions. Tris-Gly SDS-PAGE 12% acrylamide plus Urea 3M. **C,** Analysis of thylakoid phosphoproteins using anti-phospho-threonine antibody (Cell Signaling). Samples used were collected from solubilized thylakoids from dark adapted leaves or treated with HL as shown in A.

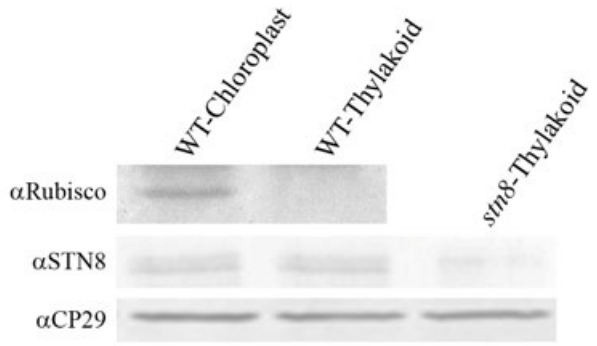

Figure 4: Subcellular localization of STN8 kinase in rice. Determination of STN8 localization by immunoblot analysis on isolated chloroplasts or thylakoids. Chloroplasts and thylakoids were isolated from dark adapted WT rice leaves. Immunoblot analyses were performed using anti-Rubisco, anti-STN8 and anti-CP29 antibodies. The presence of Rubisco reflects the presence of stromal components, while CP29 is a marker for thylakoid complexes. Thylakoids from *stn8* plants were used as a control for reliable STN8 detection, because of the presence of an aspecific band with similar molecular weight. One microgram of Chls per lane.

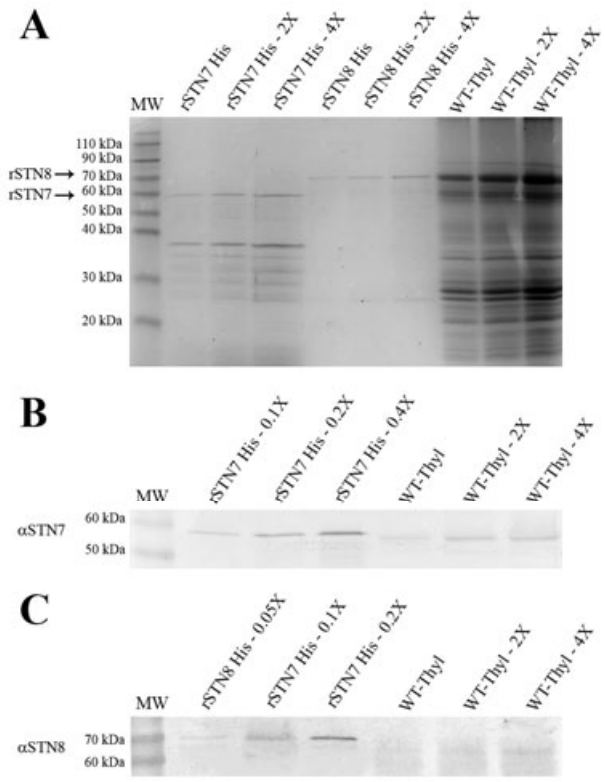

Figure 5: Determination of STN7 and STN8 kinases relative amount in isolated thylakoids. His-tagged proteins were purified by affinity chromatography and checked in Tris-Glycine SDS-PAGE. Immunoblot analyses were performed using anti-STN7 and anti-STN8 antibodies on these fractions and on thylakoid (WT-Thyl) samples isolated from wild-type plants. 0.75, 1.5 and 3 micrograms of Chls per lane were respectively loaded for WT Thyl, WT Thyl – 2X and WT Thyl – 4X. Quantification analysis was performed with a second independent preparation of thylakoids giving similar result.

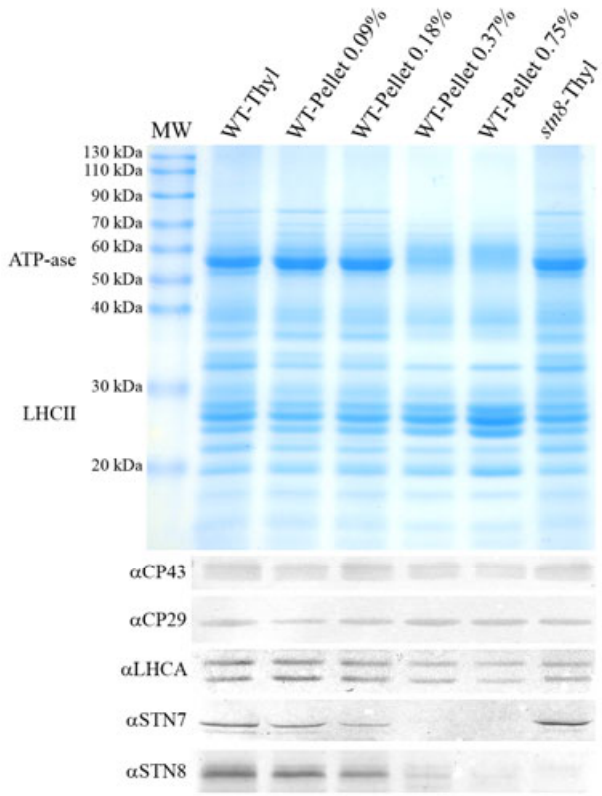

Figure 6: Distribution of STN8 kinase in thylakoid membranes. Fractionation of WT thylakoid membranes as previously reported (Morosinotto et al., 2010) through solubilization with increasing amounts of α-dodecylmaltoside (Pellet-X%, where X% represents the final concentration of the detergent), followed by centrifugation for separating solubilized membranes from unsolubilized ones. The obtained fractions were used for the determination of STN8 and STN7 kinase localization in thylakoid membranes, using the analyses with antibodies against CP43, CP29 and LHCA as indicators for grana or stroma exposed membranes enrichments. Immunoblot analyses was performed using anti-STN7 and anti-STN8 antibodies. 1.5 micrograms of Chls per lane. Fractionation analysis was repeated giving similar result.

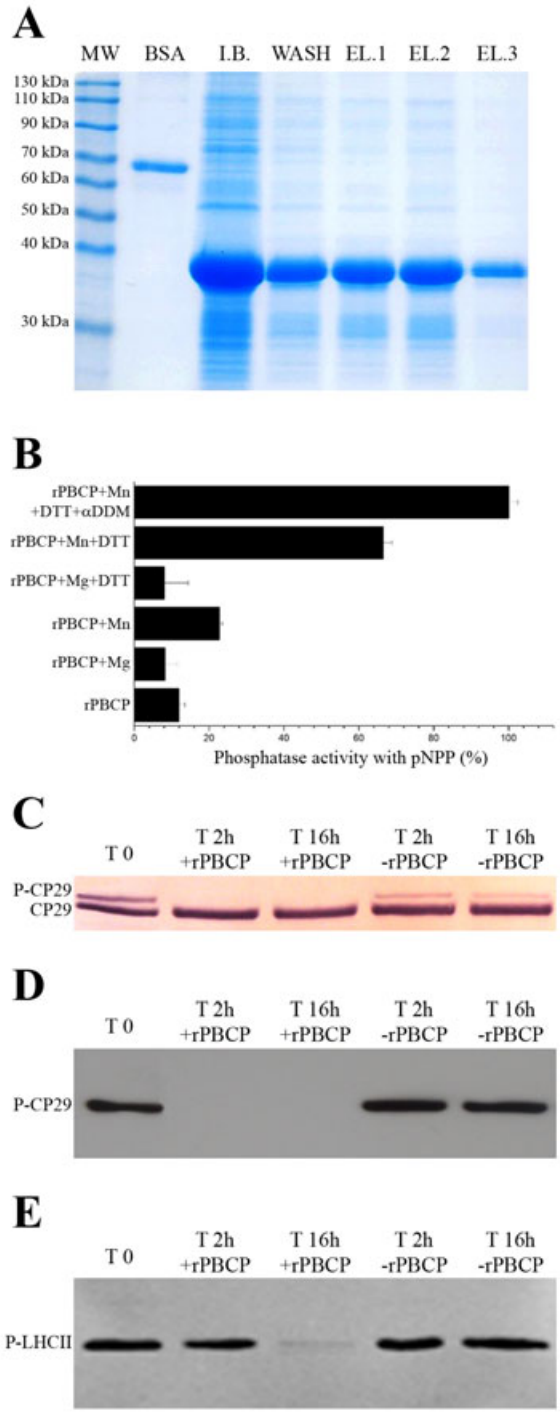

Figure 7: Recombinant PBCP of rice is able to dephosphorylate P-CP29. A, SDS-Page analysis of fractions resulted from affinity chromatography for purification of recombinant PBCP-HisTag in denaturant condition. 1 microgram of bovin serum albumin was loaded as a reference. **B,** *In vitro* spectrophotometric assay of recombinant PBCP activity upon in vitro refolding. The dephosphorylation of pNPP was measured by the increase in absorption at 405 nm in 1-mL reaction volumes containing 1 microgram recombinant PBCP, 300 mM NaCl, 50 mM HEPES, pH 8.0, 10 mM pNPP, and the additives indicated. Error bars represent the SD of triplicate measurements. **C,** Thylakoid membranes were collected from high-light treated leaves of wild-type plants. Samples (1 microgram of Chls) was finally incubated for 2 hours/ovenight in the presence/absence of purified recombinant PBCP (1 microgram). Immunoblot analysis was performed using anti-CP29 antibody. **D,** Monomeric antenna complexes were purified by sucrose gradient fractionation of solubilized thylakoids used in C. Samples (0.25 micrograms of Chls) was treated as above. Immunoblot analysis was performed using anti-phosphothreonine antibody. **E,** Trimeric LHCII complexes were purified as in D, using thylakoids isolated from leaves treated with PSII-enriched light. Samples (0.25 micrograms of Chls) was treated as above. Immunoblot analysis was performed using anti-phosphothreonine antibody.