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Regulation of Thylakoid Protein Phosphorylation during State Transition and Photosystem II Supercomplex Mobilization in Rice

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The Graduate School

Pusan National University

Department of Integrated Biological Science

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ABBREVIATIONS

ATP	Adenosine triphosphate
BL	Blue light
BN-PAGE	Blue-native polyacrylamide gel electrophoreses
BN-gel	Blue-native polyacrylamide gel
cDNA	Complementary DNA
D1	A PSII reaction center protein, D1
D1-C	An antibody against C-terminal of D1 protein
D1-DE	An antibody against DE-terminal of D1 protein
DAB	3, 3´-diaminobenzidine
DCMU	3-(4,4-dichlorophenyl)-1,1-dimethylurea
DNA	Deoxyribonucleic acid
ETR	Electron transport
Fm	Maximum fluorescence
Fo	Minimum fluorescence
FR	Far red light
Fv/Fm	Photochemical efficiency of PSII
GL	Growth light intensity
HL	High light intensity
LHCI	Light harvesting complex I
LHCII	Light harvesting complex II
LL	Low light intensity

NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NBT	Nitroblue tetrazolium
NPQ	Non-photochemical quenching
PAGE	Polyacrylamide gel electrophoreses
PCP	PSII core proteins
PCPP	PSII core proteins phosphorylation
PCR	Polymerase chain reaction
PQ	Plastoquinone
PQH_2	Reduced form of plastoquinone
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive oxygen species
RT-PCR	Reverse transcript PCR
SDGU	Sucrose density gradient ultracentrifugation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoreses
SOD	Superoxide dismutase
SOSG	Singlet oxygen sensor green
T-DNA	Transfer DNA
2D	Two Dimensional

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Regulation of Thylakoid Protein Phosphorylation during State Transition and Photosystem II Supercomplex Mobilization in Rice

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ABSTRACT

Recent research identified the role of STN8 kinase for phosphorylation of PSII core proteins such as D1, D2, CP43, PsbH, TSP9, CaS and STN7 kinase for not only phosphorylation of LHCII protein but also PSII core proteins and state transition in Arabidopsis. However complete inhibition of PSII core protein phosphorylation was only observed in the Arabidopsis *stn7/stn8* double mutant. Hence, the elucidation of specific roles of STN8 and STN7 kinases has been identified by using T-DNA insertional knock-out mutant of the rice (*Oryza sativa*) STN8 gene, *osstn8*, STN7 gene, *osstn7* and PPH phosphatase gene, *ospph*. Here,

osstn8 and *osstn7* mutants has been used to clarify the role of PSII-LHCII protein phosphorylation in regulation of PSII repair cycle, mobilization of PSII supercomplex, state transition.

In the *osstn8* mutant, PSII core protein phosphorylation (PCPP) was significantly suppressed and the grana were thin and elongated. During high light (HL) illumination, PSII was strongly inactivated in the *osstn8* mutants, yet the D1 protein was degraded more slowly than in wild type, and the mobilization of the PSII supercomplex from the grana to stroma lamellae for repair was also suppressed. Taken together, these data show that the absence of STN8 gene is sufficient to abolish PCPP in *osstn8* mutants to produce all of the phenotypes observed in the double mutant of Arabidopsis, indicating the essential role of STN8-mediated PCPP in PSII repair. Using both histochemical and fluorescence probes, ROS production, including superoxide and hydrogen peroxide production was increased in *osstn8* mutant's leaves during HL illumination. When superoxide dismutase was inhibited, superoxide production was increased, indicating that superoxide production is the initial event prior to hydrogen peroxide production. However, singlet oxygen production was not different between WT and *osstn8*. In addition, PSII-driven superoxide production was more in the thylakoid membranes as well as in isolated PSII and PSII-LHCII supercomplex of *osstn8*. PSII and PSII-LHCII supercomplex of *osstn8* leaves showed strong oxidation of proteins under HL illumination. These results suggest that superoxide is the major form of ROS produced in the *osstn8* mutant, and that the damaged PSII in the supercomplex is the primary source of superoxide production during HL illumination. Apart from this, the specific kinase and phosphatase for reversible phosphorylation of CP29 (CP29-P) was still unknown. Therefore T-DNA insertion knock-out mutant of STN7 and STN8 kinase as well

as PPH phosphatase mutants were used to confirm it. CP29-P was observed in WT, *osstn8* complement, *osstn7* and *ospph* mutant but not in *osstn8* mutant during high light and light chilling treatment. However dephosphorylation of CP29-P was observed in all genotypes during dark incubation. The phosphorylation and dephosphorylation of LHCII protein was blocked in *osstn7* mutant and *ospph* mutant, as a consequence, state transition was strongly impaired. Mobilization of PSII supercomplex was observed in *osstn7* mutant during high light illumination but it was suppressed in *ospph* mutant. This result proposed that dephosphorylation of LHCII protein was also involved for mobilization of PSII supercomplex. In control condition, the relative change in maximal fluorescence during state transition, ΔF was similar in WT and *osstn8* mutant and mobilization of PSII supercomplex was not observed but ΔF become negligible in *osstn8* during high light illumination and mobilization of PSII supercomplex was only observed in WT. Upon high light illumination, CP29-P was observed in PSI complex in WT but not in *osstn8* mutant. Therefore it is concluded that lack of CP29-P also impaired high light induced state transition in *osstn8* mutant of rice.

GENARAL INTRODUCTION

Chloroplast and thylakoid membrane

Green plant cell contains a unique cell organelle known as chloroplast where the process of photosynthesis takes place. Chloroplasts are semi-autonomous organelles of endosymbiotic origin that are found in all plants and algae. Chloroplasts belong to the diverse family of plastids. There are several types of plastids that has specialized roles and are derived from undifferentiated proplastids. The most important types of plastids are chloroplasts, which contain chlorophyll and conduct photosynthesis, leucoplasts, that does not have chlorophyll and are found mostly in roots and non-photosynthetictissue and chromoplasts which are responsible for the coloring of fruits and flowers. Furthermore, plastids are also involved in gravity perception and the opening and closure of stomata (Wise and Hoober, 2007). A typical structure of chloroplast that contains grana and stroma is shown in (Fig. 1).

The thylakoid membranes have a very distinctive heterogeneous membrane system consisting of appressed regions called the grana, and non-appressed regions connecting these stacks called the stroma lamellae. Most of the protein complexes are found in the thylakoid membrane (Fig. 2). The components of the packed grana membranes facing towards the stroma are known as grana end membranes, and the turns of each granum are known as margins (Albertsson, 2001; Allen and Forsberg, 2001; Mustárdy and Garab, 2003). The thylakoid membranes enclose the inner soluble space known as the lumen, which is a compartment that has recently been found to have a complex proteome in itself

(Kieselbach et al., 1998). The design of the grana structure has evolved to optimize the photosynthetic process, which allows plants to adapt at fluctuating light conditions (Chow et al., 2005a; Hausler et al., 2009). Grana are found in all higher plants, while most algae and cyanobacteria have very little, or totally lack grana structure (Anderson et al., 2008).

The formation and maintenance of grana in chloroplasts of higher plants is a versatile process controlled by an intricate interplay of several physiochemical forces. These forces are Van der walls attraction, entropy, electrostatic attraction and repulsion (phosphorylation), hydration repulsion and steric hindrance (Sculley et al., 1980; Chow et al., 2005b). Furthermore, in the *hcf136* mutant of Arabidopsis, which is defective in photosystem II (PSII) assembly, the thylakoid grana is severely enlarged and extended throughout the chloroplast (Meurer et al., 1998). A recent study of chlorophyll *b* less mutants established that light harvesting complex II (LHCII) complexes also play an important role in the thylakoid structure. This mutant showed less negatively charged and irregularly stacked thylakoids when compared to wild type membranes (Kim et al., 2009). These studies illustrate the importance of protein complex composition in the maintenance of the grana structure.

Photosystem I and Photosystem II

There are two photosystems are found within the thylakoid membranes of the chloroplast (Fig. 2). These are known as photosystem I (PSI) and Photosystem II (PSII). PSI optimally absorbs photons of a wavelength of 700 nm and PSII optimally absorbs photons of a wavelength of 680 nm. Photosystem II uses light energy to oxidize two molecules of water into one molecule of molecular oxygen.
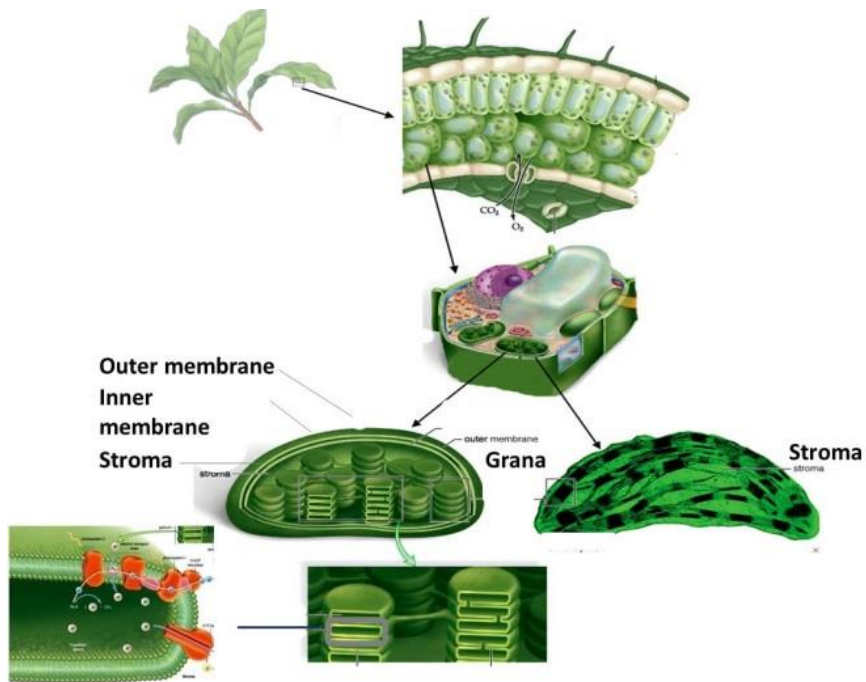
Figure 1. Typical structure of chloroplast and thylakoid membrane. Chloroplasts are green organelles found in plant and algae cells. Chloroplasts have an endosymbiotic origin, can divide and reproduce within plant cells, and have their own ribosomes and DNA. The inner membrane of the chloroplast, called the thylakoid membrane, is folded and stacked to form grana (disc-shaped structure.
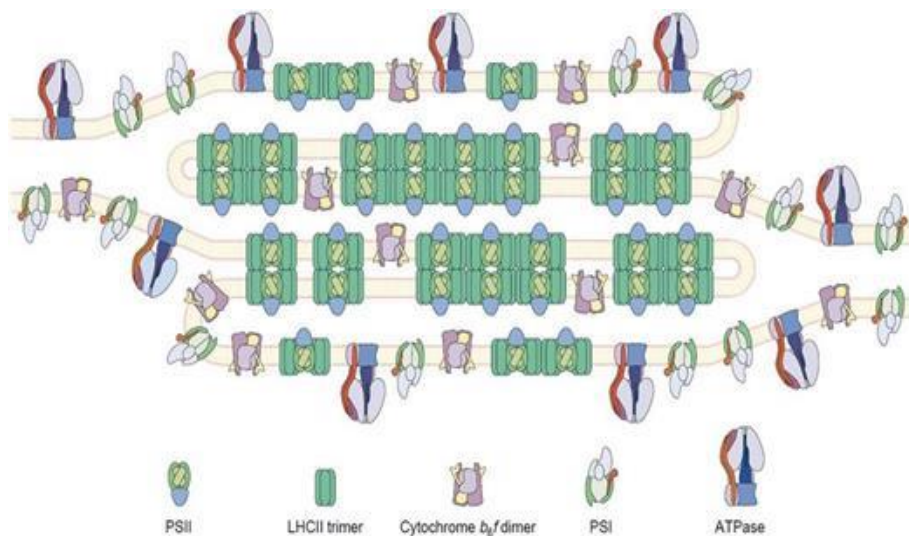
Figure 2. Molecular recognition and steric hindrance determine both the topography of thylakoid stacks and lateral heterogeneity in the distribution of photosystems I and II. A high affinity of light-harvesting complex II (LHCII) for itself, both laterally and transversely, causes LHCII-rich regions of thylakoid to become tightly appressed, forming grana. Protein complexes that contain extended, stromal-phase projections into the aqueous phase are thereby excluded from the interior of grana stacks: these are photosystem I and the coupling ATPase. The lateral separation of photosystem I and photosystem II arises from the interaction of photosystem II with the membrane-'adhesive' antenna complex, LHCII. Photosystem I is excluded from the resulting, appressed, photosystem II-rich domains by means of steric hindrance. This figure was adopted from Allen and Forsberg (2001).

During the electron transport process, a proton gradient is generated across the thylakoid membrane. This proton motive force is then used to drive the synthesis of ATP. This process requires PSI, PSII, cytochrome bf, ferredoxin-$NADP^+$ reductase and chloroplast ATP synthase. Photosystem II transfers electrons from water to plastoquinone and in the process generate a pH gradient. Plastoquinone (PQ) carries the electrons from PSII to the cytochrome bf complex. Plastoquinone is an analog of Coenzyme Q. The only differences are the methyl groups replacing the methoxy groups of Q and a variable isoprenoid tail. Plastoquinone can functions as a one or two electron acceptor and donor. When it is fully reduced to PQH_2 it is called plastoquinol. The core of this membrane protein is formed by two subunits D1 and D2.

Photosystem I catalyze the final stage of light reaction. This protein complex has two main components forming its core, psaA and psaB. These two subunits are quite larger than that the core components of PSII and the bacterial photosystems. PSI transfers the electrons across the membrane and reduces $NADP^+$ to form NADPH. NADPH is then used as reducing power for the biosynthetic reactions. The proton-motive force generated by linear electron flow from PSII to PSI powers ATP synthesis by F1Fo-complex. To synthesize ATP, photosynthesis provides an alternative route through which light energy can be used to generate a proton gradient across the thylakoid membrane of chloroplasts. This second electron path, driven by PSI only, is the cyclic electron flow, and it produces neither O_2 nor NADPH. Electrons from PSI can be recycled to plastoquinone, and subsequently to the cytochrome b6f complex (Heber and Walker, 1992; Joliot and Joliot, 2002). Such a cyclic flow generates pH and thus ATP without the accumulation of reduced species. The role of cyclic electron flow is less clear than linear, while it is proposed, that the linear flow itself cannot maintain the correct ratio of ATP/NADPH production. The absence of cyclic flow

will ultimately lead to excessive accumulation of NADPH in the stroma and thereby, its over-reduction (Munekage et al., 2004).

Photosystem II proteins phosphorylation in chloroplast

Chloroplast protein phosphorylation plays the diverse role in regulating cellular functions for signal transduction. It is estimated that around one third of the all eukaryotic proteins are subjected to reversibly phosphorylate. The reversible protein phosphorylation is dependent on protein kinases and phosphatases. Phosphorylation of thylakoid proteins is different between mesophyll cell and bundle sheath (Fristedt et al., 2012). Different protein kinases and phosphatases were identified according to their presence of catalytic domain for phosphorylationa and dephosphorylation (Schenk and Snaar-Jagalska, 1999). The reversible phosphorylation of thylakoids proteins regulates the migration of LHCII (Haldrup et al., 2001) and turnover of PSII (Rintamäki et al., 1997) which balance the absorbed excitation light energy between the two photosystems.

Recent data of phospho proteomices suggested that thylakoid function is regulated by a complex phosphorylation network in which one kinase may require for phosphorylation of several substrates and one substrate is phosphorylated by many kinases at their different amino acid sequences (Lohrig et al., 2009). Previously, the two thylakoid kinases STN8 and STN7 (ortholog of the Stt and of *Chlamydomonas reinhertii*) were identified in *Arabidopsis* (Bellafiore et al., 2005). In addition, thylakoid protein kinase (TAK) which had similarities to animal cell receptor known as transforming growth factor β receptor was identified in Arabidopsis (Snyders and Kohorn, 1999). The phosphorylation of those proteins such as PSII core proteins D1, D2, CP43, PsbH

and TSP9 and peripherial major harvesting protein LHCII as well as minor light harvesting protein CP29 and some proteins of PSI like PsaP and PsaD have been identified (Hansson and Vener, 2003). Several thylakoid phospho proteins and their kinase have been observed in different plant species as summarized by Pesaresi et al. (2011).

STN8 kinase mediated photosystem II protein phosphorylation

PSII complex is multi protein complex where most of the proteins are phosphorylated at different environmental conditions. It is known that, phosphorylation of D1, D2 protein increase with increasing light intensity, in constract to LHCII protein phosphorylation that decreased with increasing light intensity (Rintamäki et al., 1997). In *Arabidopsis thaliana*, STN8 kinase was identified for PSII core protein (D1, D2, CP43 and PsbH) phosphorylation under high light illumination without disturbing LHCII protein phosphorylation and state transition (Bonardi et al., 2005; Vainonen et al., 2005; Tikkanen et al., 2010). The PSII core proteins D1, D2 phosphorylated at low and high light condition and controlled by STN8 kinase (Bonardi et al., 2005). In addition CP43, PsbH, CaS proteins are also phosphorylated by STN8 kinase (Reiland et al., 2009). The activation of this kinase is depends on different environmental condition in the leaf. STN8 is activated at low light and high light via reduced plastoquinone pool although light dependent phosphorylation of CaS is connected by Ca^{2+} signaling pathway by STN8 kinase (Schönberg and Baginsky, 2012). Apart from this, the activation of this kinase is regulated by ferredoxina and thioredoxin system. Under high light, electron flow from PSII through PSI generates more reduced ferredoxin and thioredoxin that activate STN8 kinase and inactivate PSII core protein phosphatase (PBCP) (Cui et al., 2014).

PSII core protein phosphorylation is essential for PSII repair cycle and inhibition of reactive oxygen species formation (Tikkanen et al., 2008). While PSII core protein phosphorylation controlled the macroscopic folding of thylakoid membrane (Fristedt et al., 2009), LHCII and CP29 protein phosphorylation is responsible for state transition (Rochaix, 2007, Tokutsu et al., 2009). Similarly, phosphorylation of CP29 has several roles. For example CP29 phosphorylation dissipate the excess of excitation energy and protect against photoinhibition (Bergantino et al., 1998), state transition in Arabidopsis (Tikkanen et al; 2006) and *Chlamydomonas* (Kargul et al., 2005, Tokutsu et al., 2009), induce a conformational change which modify the chlorophyll organization leading non-radioactive energy dissipation (Croce et al., 1996), modulate the spectral properties of photosystem II subunit and provide a possible mechanism for the regulation of excitation energy supply to the reaction center (Croce et al., 1996), induce disassembly of PSII-supercomplex (Fristedt et al., 2011).

STN7 kinase mediated LHCII protein phosphorylation and state transition

Previously, Stt7 in *Chlamydomonas reinhardtii* (Depège et al., 2003) and Stn7 in *Arabidopsis thaliana* (Bonardi et al., 2005) have been identified for LHCII protein phosphorylation. It is also known that high light treatment inactivates the kinase responsible for phosphorylation of LHCII through ferredoxin-thieoredoxin system (Hou et al., 2003). Mobilization of LHCII protein at preferential excitation of PSI in state 1 and PSII in state 2 is depicted in Fig. 3. The two conserved cysteine could therefore be the targets of this redox system which appears likely that activity of Stn7/Stt7 kinase is regulated by a complex networking involving cooperative redox control by PQ and cytochrome b6f

complex as well as by the ferredoxin-thioredoxin system in the stroma (Lemeille and Rochaix, 2010). In addition, the phosphorylation of LHCII is induced by reactive oxygen species (ROS) and then the phosphorylated LHCII moves to PSI, into the repair cycle of damaged PSII. The ferredoxin and thioredoxin system may play a possible central role in the phosphorylation regulation on LHCII dissociation (Cui et al., 2014).

However, the exact nature of activation and deactivation of kinase by reduced and oxidized PQ pool is unknown (Joshi et al., 2011). Over the years, there have been arguments regarding which LHCII proteins are involved in state transitions and a couple of theories were put forward (Kouřil et al., 2005; Damkjaer et al., 2009; Tokutsu et al., 2009). One theory suggests the role of LHCII trimer in state transitions and the other holds CP29 responsible. However these concepts were put forward by using two different model organisms: *Chlamydomonas reinhardtii* and *Arabidopsis thaliana* suggesting the existence of two completely different modes of state transitions of the three LHCII trimers, S, M and L, the trimer which is involved in state transitions has been a mystery until recently. Initially it was thought that LHCII-M might be involved in state transitions as it is the trimer which could be easily dislocated from the supercomplex (Allen, 1992). However, the failure to detect Lhcb3 in the stroma lamella discarded this hypothesis as it is the main protein in LHCII-M. Also trimer S is very strongly bound to PSII core and therefore its involvement in state transitions is quite unlikely. The next suitable candidate for state transitions is LHCII-L. However, the inability to isolate a stable LHCII-PSI supercomplex hampered the confirmation of this theory until Galka et al. (2012), who reported the isolation and characterization of PSI-LHCII supercomplex from *Arabidopsis thaliana* and *Zea mays*. They showed that the Lhcb protein composition of PSI-LHCII is different from that of LHCII-S, LHCII-M and CP29; although they

couldn't directly prove that it is composed of LHCII-L as stable isoform of LHCII-L has not been successfully accomplished.

PPH phosphatase mediated dephosphorylation of LHCII and state transition

The activation of kinase in the thylakoid membrane is controlled by the redox state of the plastoquinone (PQ) pool (Vener et al., 1997; Zito et al., 1999). When light conditions favor the activity of PSII, reduction of the PQ pool activates the STN7 kinase and causes a transition to state 2. The LHCII antenna protein is phosphorylated (Allen, 1992) and move toward PSI (Lunde et al., 2000). This process is reversible; because when PSI is more active and the plastoquinone pool is oxidized, the LHCII antenna is dephosphorylated and move back toward with PSII. Little is known about the molecular nature of the phosphatases involved in state transitions (Aro and Ohad, 2003). Dephosphorylation of LHCII proteins was observed with isolated thylakoids, indicating that at least a portion of the phosphatase is membrane associated (Bennett, 1980). It was further shown that thylakoid protein phosphatases are redox independent and kinetically heterogeneous (Silverstein et al., 1993). A 29 kDa stromal protein phosphatase was shown to act on dephosphorylation of LHCII in vitro (Hammer et al., 1997). However, it was not clear whether this protein functions in the dephosphorylation of LHCII in vivo or not.

When PQ is oxidized and LHCII kinase is inactivated then LHCII protein become dephosphorylated by the action of unknown protein phosphatase. For several years, many scientists attempted to identify LHCII protein phosphatases. And finally TAP38/PPH1 phosphatase that was required to dephosphorylate LHCII protein has been identified (Pribil et al., 2010; Shapiguzov et al., 2010).
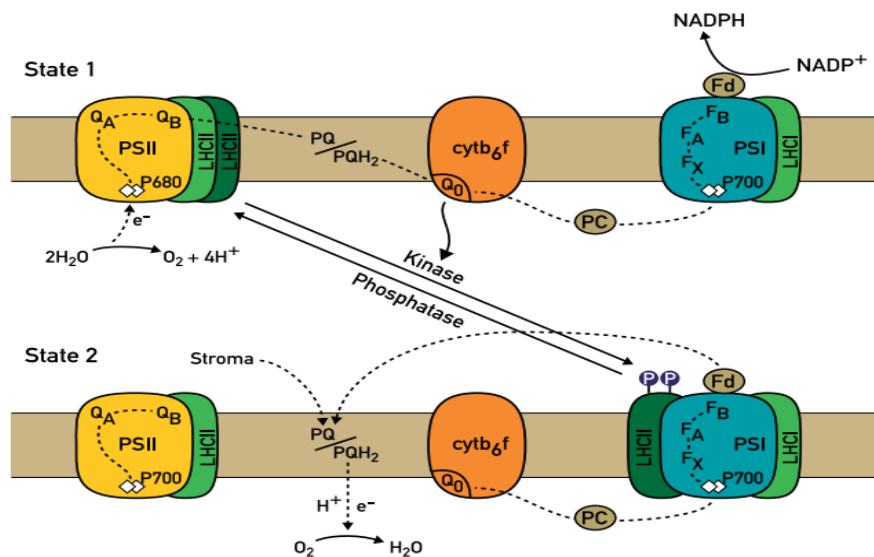
Figure 3. Scheme of photosynthetic complexes in the thylakoid membrane and state transitions. State transitions are determined to a large extent by the redox state of the PQ pool. Preferential excitation of PSI leads to the oxidation of the PQ pool and to state 1. In state 1 the mobile light-harvesting antenna is bound to PSII. Preferential excitation of PSII relative to PSI leads to a reduced state of the plastoquinone pool and thus to the docking of plastoquinol to the Qo site of the cytochrome *b6f* complex. This activates the LHCII kinase and leads to the displacement of phosphorylated LHCII from PSII to PSI (state 2). This figure was adopted from Rochaix (2007).

In addition, another protein phosphatase, "Photosystem II core protein phosphatase (PBCP) phosphatase" has been identified that required for dephosphorylation of PSII core proteins but not LHCII in Arabidopsis (Samol et al., 2012).

PSII-LHCII supercomplex mobilization

PSII-LHCII supercomplex is the functional form of PSII which are organized in the appressed region of grana membranes. When plants are illuminated with strong light, the mobilization of PSII-LHCII supercomplex from grana to stroma lamella is observed probably as a photoprotective mechanism. When PSII proteins are phosphorylates then PSII remodeling observed and they move towards stroma (Fig. 4, 5). Although the exact mechanisms of this high light induce mobilization of PSII-LHCII supercomplex is still unclear, several recent reports have suggested that the mobilization of PSII-LHCII supercomplex is controlled by the phosphorylation of PSII core proteins, especially the phosphorylation of PSII core antenna protein CP43 by STN8 kinase and the phosphorylation of LHCII proteins by STN7 kinase (Tikkanen et al., 2008; Dietzel et al., 2011). Therefore this process can divided into two steps: an initial step until CP43 phosphorylation and the next step for actual dissociation and movement of the supercomplex to granal margins or to stroma lamella. Then the damaged PSII core by high light illumination will be dephosphorylated and degraded for PSII repair (Tikkanen et al., 2008). Recently the mobilization of CP29 from PSII-LHCII supercomplex to PSII monomers and dimers was reported to occur after its phosphorylation during high light illumination, and CP29 phosphorylation may induce the disassembly of the PSII supercomplex in

Arabidopsis (Fristedt et al., 2009). The phosphorylation of CP29 is also proposed to be essential for state transitions in *Chlamydomonas reinhardtii* (Tokutsu et al., 2009). Therefore, it has been postulated that the phosphorylation of PSII proteins accelerate its mobilization during light illumination. Apart from this, the protonation of the PsbS protein also facilitates the migration of the PSII-LHCII proteins along the thylakoid membrane for non-photochemical quenching during light illumination (Kereiche et al., 2010). Hence it has been assumed that phosphorylation of PSII core proteins (Baena-González et al., 1999), LHCII proteins (Tikkanen et al., 2008) as well as phosphorylation of PSII core antenna protein CP43 was an important prerequisite for the release of PSII supercomplex (Dietzel et al., 2011), and furthermore, the protonation of the PsbS protein (Kereïche et al., 2010) has been shown to have a role in facilitating the migration of the PSII-LHCII proteins along the thylakoid membrane during light illumination (Fig. 5).

Photosystem II turnover and repair

An earlier study on Arabidopsis mutants *stn7*, *stn8* and *stn7x8* double mutant concluded that PSII protein phosphorylation was not essential for PSII repair (Bonardi et al., 2005), recently PSII of oxygenic photosynthetic machinery is constantly damaged by light and its repair is coordinated by phosphorylation of PSII core proteins by STN8 kinase and LHCII protein by STN7 kinase (Tikkanen and Aro, 2012). There are several physiological and biochemical cascades involved for phosphorylation and PSII repair cycle in lower and higher plants. In PSII repair cycle several separate mechanisms are mainly involved for its repair mechanism in the presence of light.
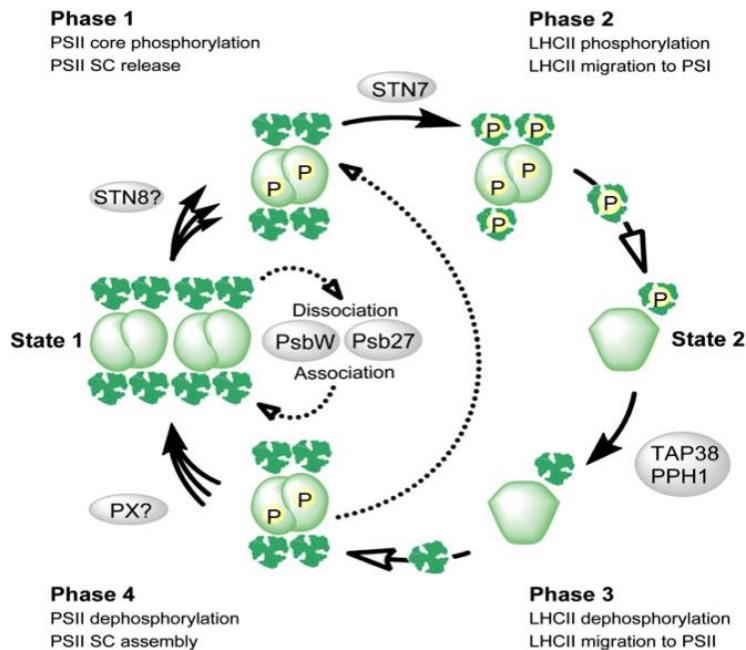
Figure 4. Schematic model for initiation and processing of PSII supercomplex remodeling and state transitions in Arabidopsis. The model depicts the reversible involvement of PSII supercomplex remodeling (including the generation of megacomplexes) upon a transition from state 1 to state 2 (given in bold letters) and vice versa in a continuous cycle that can be separated into four phases each with a specific function (indicated in the respective corners of the figure). The model aims to represent the basic principle rather than being complete in all three-dimensional details of the complexes. Entangled green ovals indicate PSII dimers, surrounding dark-green structures the LHCII trimers, and a green hexagon PSI. Phosphorylation of the respective structures is given by a P in yellow circles. Arrows between the respective structures mark the sequence of events. White arrowheads indicate movement of LHCII. Tripartite arrows represent the sequential generation or release of the various organization steps of PSII from dimers up to megacomplexes. This figure was adopted from Dietzel et al. (2011).

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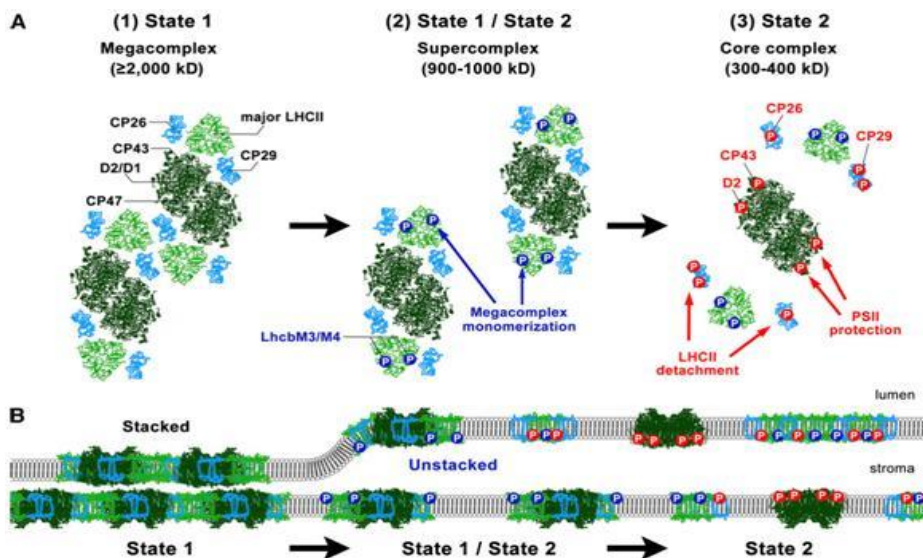
Figure 5. A proposed model for the remodeling of PSII during the transition from state 1 to state 2. (**A**) A top-down view from the stromal side of the membrane showing the division of the PSII megacomplex and detachment of peripheral antenna: (1) unphosphorylated LHCIIs stabilize the megacomplex; (2) the phosphorylation of LHCII type I in the major LHCII trimer triggers the division of themegacomplex, resulting in discrete C2S2 supercomplexes; (3) the phosphorylation of CP26 and CP29 as well as PSII core subunits D2 and CP43induces the undocking of most LHCIIs from the PSII core complex. (**B**) A side-view of the membrane plane showing alterations in the thylakoid ultrastructure: (1) thylakoids are more stacked in State 1 (left); (2) after membrane unstacking, which is likely caused by the phosphorylation of LHCII type I, more LHCII proteins, including CP26 and CP29 and the core proteins CP43 and D2 protein, are phosphorylated (middle), leading to the undocking of most peripheral antenna proteins from PSII (right). This figure was adopted from Iwai et al. (2008).

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In the presence of light, the damaged PSII is replaced by degradation of damaged PSII by FtsH and Deg proteases and replaced newly synthesized PSII (Komenda et al., 2012). The major target to damage PSII is D1 protein of PSII reaction center (Nixon et al., 2010). The highly hydrophobic D1 protein contain five transmembrane helices with N-terminal and C-terminal tails exposed to the stroma and thylakoid lumen (Loll et al., 2005, Umena et al., 2011). To achieve this goal different steps are involved for PSII repair cycle in which damaged D1 protein can replace by newly synthetized D1 protein. For the turnover of the damaged PSII core proteins, in particular the D1 protein, they must go through the following highly organized steps of PSII repair cycle: (1) Phosphorylation and photodamaged of PSII proteins, (2) monomerization, migration and disassembly of photodamaged PSII proteins, (3) dephosphorylation and degradation of PSII proteins, (4) de novo biosynthesis of D1 protein (5) insertion of newly synthesized D1 protein in PSII complex and (6) PSII reassembly and functional PSII (Nath et al., 2013a). To achieve the goal of PSII repair cycle different protein and phosphorylation of PSII protein are involved. Since PSII consists of more than 20 subunits and functional PSII must be maintained which is PSII repair where damaged D1 protein is degraded by proteases and replaced by newly synthesized D1 protein (Baena-González and Aro, 2002) and different auxillary proteins also involved in the biogenesis and maintenance of PSII (Mulo et al., 2008). In addition, Psb27 is also essential for PSII repair in thermophilic cyanobacterium (Grasse et al., 2011). Thus, PSII repair cycle is one of the complicated biochemical mechanisms in thylakoid membranes (Fig. 6) and to elucidate this concept here, this dissertation shows protein phosphorylation and its significance to maintain the functional PSII during light acclimation.

Thylakoid protein phosphatases and proteases for PSII repair

It has been proposed that phosphorylated form of D1 protein is poor substrate for its proteolytic degradation thus dephosphorylation of D1 proteins seems crucial steps for its degradation in stroma (Sun et al., 2007). Recently, PBCP phosphatase was identified for dephosphorylation of PSII core protein (Samol et al., 2012). Previously D1 degradation and PSII repair cycle has been reported (Tikkanen et al., 2008). Different isoforms of Deg proteases has been also reported for initial degradation of D1 protein (Kapri-Pardes et al., 2007).

Initially for the degradation of damaged D1 protein, Deg2 cleavage between D and E helices (DE loop) into 23 kDa and 10 kDa in N-terminal and C-terminal fragments (Huesgen et al., 2005), Deg1 cleavage between A and B helices (AB loop) into 10 kDa and 23 kDa in N-terminal and C-terminal fragments (Huesgen et al., 2005), Deg5 and Deg8 involve to cleavage between C and D helices (CD loop) into 18 kDa and 16 kDa in N-terminal and C-terminal fragments (Sun et al., 2007). Besides degradation of D1 protein, it was observed that photodamaged D2, CP43 and CP47 also cleaved initially by Deg7 (Sun et al., 2010a). Based on this study, FtsH proteases further degrade the D1 protein which was initially degraded by Deg proteases. There are several isoforms of FtsH proteases has been identified in Arabidopsis which consists FtsH,1 FtsH5, FtsH6, FtsH7, FtsH8, FtsH9 (Sakamoto et al., 2003), and FtsH2 (Takechi et al., 2000) for D1 degradation. Later Huesgen et al. (2009), suggested that the cleaved D1 protein by Deg and other proteases follow the progressive degradation in its N-terminal and C-terminal by FtsH proteases. Thus this evidence suggests that FtsH proteases are responsible only for degradation of D1 fragments. In PSII, D1 is the primary target for photodamage so that its degradation and repair is essential. It was observed that not only D1 protein but also D2, CP43, CP47, LHCII and PsbH

proteins also damaged during light condition and those damaged protein were also replaced in PSII (Aro et al., 2005, Sun et al., 2007).

Recent data specifically suggest ATP dependent FtsH metalloprotease and ATP dependent Deg endoprotease have significant role for D1 degradation in the thylakoid membrane of higher plants (Kato and Sakamoto, 2009; Komenda et al., 2012) as well as in vivo cooperative degradation of D1 protein, in which Deg cleave assists FtsH processive degradation under photoinhibitory condition (Kato et al., 2012). Based on these data, it can assume that, photodamaged PSII proteins degraded by Deg and FtsH proteases and most of those proteins are phosphoproteins. The question arise whether dephosphorylation of D1 protein is essential for its degradation or not and proteases have that ability to degrade phosphorylated form of D1 protein?, to get the clear concept for this mechanism more effort is essential. In addition, there are other proteases that exist which can degrade photodamaged PSII in appressed grana region. Because if phosphorylated D1 protein starts degradation in PSII-LHCII supercomplex then there may have some reasons (1) there are another phosphatases and proteases are localized in the appressed region of grana where PSII-LHCII supercomplex reside (2) completely dephosphorylation of D1 protein is not required for its complete degradation, (3) there are some proteases which can degrade phosphorylated D1 protein in PSII supercomplex and (4) phosphorylation, mobilization and degradation process occur simultaneously.
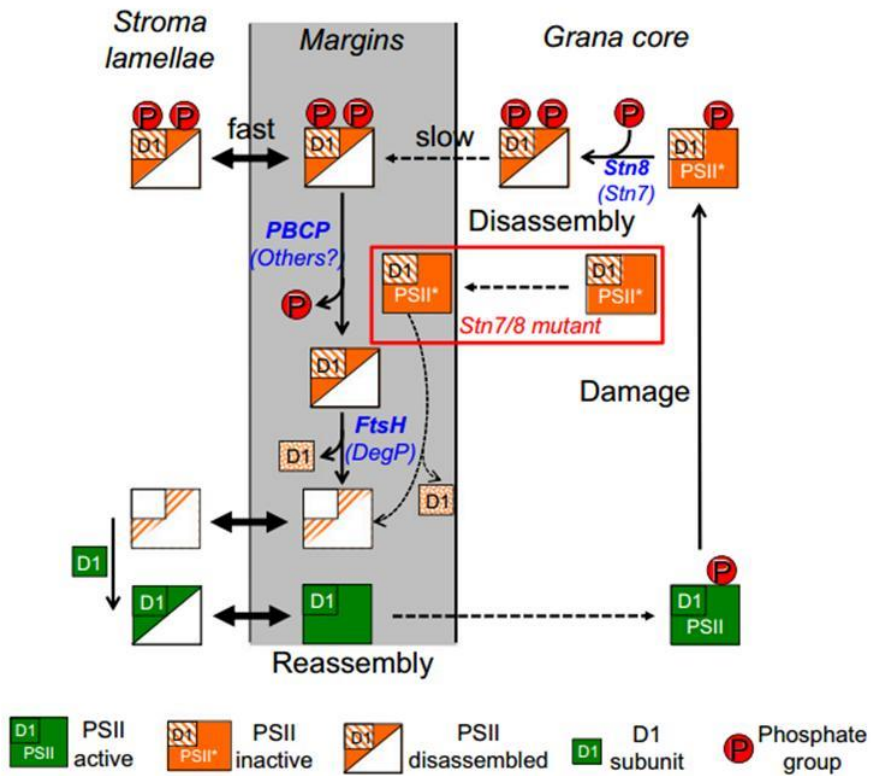
Figure 6. Scheme of the PSII repair cycle showing temporal and spatial segregation of the enzymes and the reactions they catalyze. The thylakoid subdomains-grana core, grana margins, and stroma lamellae-are demarcated. A key representing various assembly states and modifications of PSII is given below the figure. This figure was adopted from Puthiyaveetil et al. (2014).

Reactive oxygen species (ROS) and PSII repair

Under many biotic and abiotic stress conditions, an imbalance between ROS generation and scavenging occurs, and the accumulating ROS cause damage to cells near their generation sites (Hideg et al., 2002). Even though ROS are scavenged by diverse enzymatic and non-enzymatic antioxidative defense substances (Mittler, 2002), the levels of ROS may rise rapidly following environmental changes (Blokhina et al., 2003). The main source of ROS in chloroplasts is the electron transport chain and the generation site for each ROS differs depending on the stress applied (Laloi et al., 2004). In the photosynthetic machinery, the conversion of excitation energy absorbed by chlorophylls into the energy of separated charges and subsequent water-plastoquinone oxido-reductase activity are inadvertently coupled with the formation of ROS. Singlet oxygen is generated by the excitation energy transfer from triplet chlorophyll formed by the intersystem crossing from singlet chlorophyll and the charge recombination of separated charges in the PSII antenna complex and reaction center of PSII, respectively. Apart from energy transfer, the electron transport, associated with the reduction of plastoquinone and the oxidation of water, is linked to the formation of ROS including superoxide anion radical and hydrogen peroxide. PSII evolved a highly efficient antioxidant defense system to scavenge ROS and protect PSII from oxidative damage. Both the check and balance in the formation and scavenging of ROS are controlled by the energy level and the redox potential of the excitation energy transfer and the electron transport carrier, respectively (Pospišil, 2012). Since the D1 protein is the main target of photoinhibition in PSII RC, ROS can induce the specific cleavage of the D1 protein in vitro (Okada et al., 1996). Based on in vivo studies of cyanobacteria, it has also been suggested that ROS act primarily by inhibiting the synthesis of the D1protein but not by damaging PSII directly (Nishiyama et al., 2004).

When photosynthetic tissues are illuminated in excess of the energy utilization potential of carbon reduction, there is a marked decrease in photosynthetic capacity, and this over-excitation of the photosynthetic apparatus may result in damage to PSII reaction centers (Krause, 1988; Melis, 1999). However recent findings demonstrated that PSII repair process is more sensitive to environmental stress than the damage of PSII, due to inhibitory action of ROS in D1 protein synthesis (Takahashi and Murata, 2008; Nishiyama et al., 2011). In addition to other environmental stresses affecting the repair cycle of PSII, it is evident that PSII photoinhibition as such is enhanced by abiotic stresses (Nath et al., 2013a). Here, based on the mutation of *osstn8* gene in rice which causes impairment in PSII core protein phosphorylation and renders *osstn8* mutants to photooxidative damage, a new model of the PSII photoinhibition-repair cycle is proposed (Nath et al., 2013a). Accumulation of damaged D1 protein is possibly due to impaired disassembly of PSII and/or modified grana stacking in mutant plants, as reported by (Tikkanen and Aro, 2012). Accumulation of damaged PSII complexes is likely to be the main reason for ROS generation in *osstn8* mutant. ROS generation due to photooxidative damage mainly leads to: i) slow down of D1 protein degradation, and ii) inhibition of D1 protein de novo synthesis, during photoinhibition.

OBJECTIVE OF THE STUDY

The aim of this research was to investigate the role of PSII and LHCII protein phosphorylation for mobilization of PSII supercomplex and state transition in rice plant. Phosphorylation of thylakoid membrane proteins in the presence of sunlight, balance the energy between two photosystems which has major role in the process of photosynthesis. In recent years there are some controversies regarding the exact role of proteins phosphorylation in the thylakoid membrane of higher plants. STN7 and STN8 are the major thylakoid kinases to phosphorylate major LHCII and PSII core proteins respectively. Apart from this PPH phosphatase was identified to dephosphorylate major LHCII proteins. Therefore STN7, STN8 and PPH phosphatase knock-out mutants of rice were used to clarify the role of protein phosphorylation and dephosphorylation in the thylakoid membrane during light illumination.

The main objectives of this research work are given below:

- To clarify the role of PSII core protein phosphorylation for PSII-LHCII supercomplex mobilization during high light illumination.

- To clarify the role of PSII core protein phosphorylation for PSII repair during high light illumination.

- To investigate the role of PSII core protein phosphorylation to to suppress the production of reactive oxygen species (ROS) during high light illumination.

- To investigate the production site of ROS in the thylakoid membrane.

- To investigate the kinase for CP29 protein phosphorylation during high light illumination.

- To investigate the process of state transition by LHCII protein phosphorylation and CP29 protein phosphorylation during low light and high light illumination respectively.

PART I

Suppression of Photosystem II Repair by Blocking the Phosphorylation of D1 Protein in the *OsSTN8* Knock-out Rice Mutant

ABSTRACT

The role of STN8 kinase for photosystem II (PSII) core protein phosphorylation (PCPP) in PSII repair cycle that was on debate in Arabidopsis (Bonardi et al., 2005, Nature, 437: 1179-1182) was reinvestigated using a rice (*Oryza sativa*) T-DNA insertional STN8 knock-out mutant, *osstn8*. In the *osstn8* mutant, PCPP was significantly suppressed and the grana were thin and elongated. Upon HL illumination, PSII was strongly inactivated in the mutants, yet the D1 protein was degraded more slowly than in wild type, and the mobilization of the PSII supercomplex from the grana to stroma lamellae for repair was also suppressed. In addition, higher accumulation of reactive oxygen species and preferential oxidation of PSII reaction center core proteins in thylakoid membranes were observed in the mutants during HL illumination. Taken together, these data show that the absence of STN8 is sufficient to abolish PCPP in *osstn8* mutants to produce all of the phenotypes observed in the double mutant of Arabidopsis, indicating the essential role of STN8-mediated PCPP in PSII repair.

1. INTRODUCTION

Plants use light during photosynthesis to convert it into chemical energy however excessive light is harmful to plants. Even in low light, photosystem II (PSII) can be damaged in proportion to the light intensity (Tyystjärvi and Aro, 1996; Jansen et al., 1999; Oguchi et al., 2009). Damaged PSII needs to be repaired at all light intensities, and the overall level of photosynthetic activity is reduced when the rate of repair is unable to match the rate of photodamage (Prasil et al., 1992). The repair of photodamaged PSII, particularly the turnover of the D1 protein, is thought to be a key regulatory mechanism of the PSII repair cycle (Yokthongwattana and Melis, 2006). In this cycle, the damaged and phosphorylated PSII is first relocated from the grana to the stroma lamellae, where the damaged D1 protein is degraded after dephosphorylation by specific proteases (Bailey et al., 2002; Kapri-Pradesh et al., 2007; Sun et al., 2010b; Kley et al., 2011; Schuhmann and Adamska, 2012; Wagner et al., 2012; Kirchhoff, 2013a), and the newly synthesized D1 is inserted into PSII (Ohad et al., 1985; Aro et al., 1993; Rintamäki et al., 1996; Tikkanen et al., 2008; Tikkanen and Aro, 2012).

The phosphorylation/dephosphorylation cycle of PSII core proteins has been identified as one of the regulatory mechanisms responsible for protecting PSII from photoinhibition (Tikkanen et al., 2008; Frietedt et al., 2009; Goral et al., 2010; Tikkanen and Aro, 2012). A serine/threonine protein kinase, STN8, has recently been identified in *Arabidopsis thaliana* (hereafter Arabidopsis) to be specific for the phosphorylation of PSII core proteins, D1, D2, CP43, PsbH and TSP9 (Bonardi et al., 2005; Vainonen et al., 2005). *Osstn7* kinase in Arabidopsis was found to be involved not only in light harvesting complex II (LHCII) protein

phosphorylation (Bellafiore et al., 2005; Bonardi et al., 2005), but also in PSII core protein phosphorylation (PCPP) (Bonardi et al., 2005; Tikkanen et al., 2008). Complete inhibition of PCPP was therefore only observed in the *Arabidopsis stn7/stn8* double mutant (Tikkanen et al., 2008, Bonardi et al., 2005).

Recently, the role of PCPP in PSII repair cycle was questioned because complete inhibition of PCPP did not render any significant effects on PSII activity and D1 turnover rate in Arabidopsis *stn8* and *stn7*/*stn8* double mutants under high light (HL) illumination (Bonardi et al., 2005). However, under prolonged illumination of HL, PSII of the stn7/stn8 double mutants became more sensitive, and the degradation of photodamaged D1 protein was slower compared with wild-type plants (WT) (Tikkanen et al., 2008; Frietedt et al., 2009). In addition, in the double mutants the lack of PCPP prevents the disassembly of PSII-LHCII supercomplex during HL illumination, which blocks the migration of damaged PSII units from the grana to the stroma (Tikkanen et al., 2008; Tikkanen and Aro, 2012).

In thylakoid structure, PCPP facilitates this lateral mobility of membrane proteins, and the restriction in lateral migration of D1in *stn8* and *stn7/stn8* mutants of Arabidopsis led to the formation of extended appressed grana regions of thylakoids (Frietedt et al., 2009). More recently, several reports proposed the central role of PCPP in dynamic restructuring of thylakoid membranes in the light that involves expansion of granal thylakoid lumen (Kirchhoff et al., 2011) and lateral shrinkage and vertical destacking of grana (Herbstová et al., 2012; Kirchhoff, 2013b). Although the evidences for the important role of PCPP in PSII repair are accumulated, its detailed regulatory mechanisms and the regulatory networks are unclear yet, and there are several inconsistencies in the results obtained from algae, dicots and monocots (Chen et al., 2013).

In view of the above reports, here I have examined the characteristics of a T-DNA insertional STN8 knock-out mutant of the monocot rice (*Oryza sativa*), *osstn8*. In comparison with earlier findings in the stn8 mutant of dicot Arabidopsis, clear phenotypes have been observed in the *osstn8* mutants. These results indicate that the functional redundancy between STN8 and STN7 during PCPP is much lower in rice than that in Arabidopsis, and that the absence of STN8 is sufficient to abolish PCPP in *osstn8* mutants to produce all of the phenotypes observed in the double mutant of Arabidopsis, supporting the essential role of PCPP through STN8 kinase in PSII repair in rice.

2. MATERIALS AND METHODS

2.1. Plant materials and growth conditions

From a T-DNA inserted mutant pool, *OsSTN8* knock-out mutant *osstn8* was used as described in Nath et al., 2013b. Wild-type (WT) rice (*Oryza sativa* cultivar 'Hwayoung-byeo' (HW) and *osstn8* lines were used in the experiments. One-month-old plants were used to conduct the experiments that were grown in a glasshouse at $30^{\circ}C/26^{\circ}C$ with a 16/8 h day/night photoperiod. The source of growth light in glasshouse was sun light that changes from 0 to 1,200 µmol m^{-2}s^{-1} in whole year in Busan, Republic of Korea. Before performing the experiments, plants kept in rice growth chamber with constant light intensity at 100 µmol m^{-2}s^{-1} for 4-6 h for acclimation. Leaf segments were prepared and float on water then leaves were dark adapted for 6 h before light treatment. Similarly, Arabidopsis *stn7*, *stn8*, *pph1-3* and CP29 mutant (*KO-CP29*) seeds were kindly provided by professor Roberto Bassi, University of Verona, Italy and plant were grown at 22 ± 2 $^{\circ}$C at 16 h light and 8 h dark condition.

2.2. Genetic complementation of *osstn8*

The full-length *OsSTN8* cDNA was amplified by PCR using the primers forward 5'-CG<u>GGATCC</u>CG-AGAGCCCCCTCCTCTCCAT-3' and reverse 5'-G<u>ACTAGT</u>CCTTCCTCCTCCTTTTCCCTC TCTC-3' (the underlined sequences correspond to *Bam*HI and *Spe*I sites, respectively). A cDNA clone (AK287774) from Knowledge-based Oryza Molecular Biological Encyclopedia (KOME, http://cdna01.dna.affrc.go.jp/cDNA/) was used as the template. The PCR product

was cloned into the pGEM-T Easy vector (Promega, Madison, WI) and confirmed by sequencing. In accordance with Eom et al. (2011), the insert digested with *Bam*HI and *Spe*I was placed between the maize (*Zea mays*) *Ubiquitin1* (*Ubi1*) promoter and the *Nopaline Synthase* (*Nos*) terminator of the Ubi/NC4300 binary vector carrying the *Phosphomannose Isomerase* gene as a selectable marker. The resulting construct, *Ubi1::OsSTN8*, was used to transform the *osstn8* mutant as described previously (Lucca et al., 2001). The genetic complementation experiment was kindly done by Dr. Jong-Seong Jeon (Kyung Hee University). More than ten independent transgenic rice plants were obtained, among which a line with high expression of *OsSTN8* was used in this study as *osstn8* complemented line 1 (Comp-1).

2.3. Measurement of chlorophyll fluorescence

Room temperature chlorophyll fluorescence parameters including minimum fluorescence (Fo) and maximal fluorescence (Fm) from detached leaf segments were measured using PAM 2000 (Walz Effeltrich, Germany) following the manufacturer's instructions after dark incubation for 30 min. The photochemical efficiency of PSII, or Fv/Fm, was calculated using the equation, Fv/Fm = (Fm - Fo) / Fm.

2.4. Infiltration of protease inhibitors or lincomycin and photoinhibitory treatments

Leaves were chosen from WT and *osstn8* mutant rice plants, and about 2-3 cm leaf segments were prepared in water to protect against severe wounding

and used for infiltration. For infiltration of protease inhibitors, leaf segments were floated on either 10 mM phenylmethylsulfonyl fluoride (PMSF) or 10 mg/ml protease inhibitor cock tail (Sigma) solution for 6 h in darkness. For infiltration of plastid protein synthesis inhibitor, leaf segments were submerged in 3 mM lincomycin solution overnight in darkness.

For photoinhibitory treatments, leaf segments were placed under 1,500 µmol m^{-2}s^{-1} light intensity for 2 h using a metal halide lamp equipped with a water bath to reduce heat. During HL illumination, 3 mM lincomycin solution was replaced with 1 mM lincomycin to reduce the toxic effects of this agent.

2.5. Immunoblotting and Oxyblot analysis

For immunoblotting, leaf segments were dark-adapted overnight with or without 3 mM lincomycin, and then exposed either to Low light (LL) at 200 µmol m^{-2}s^{-1} or to HL illumination at 1,500 µmol m^{-2}s^{-1} for 2 h. Thylakoid membranes were isolated following the protocol described by Oh et al. (2009), and chlorophyll concentration in isolated thylakoids was measured following the method of Porra et al. (1989). Sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE) and immunoblotting were carried out as described by Towbin et al. (1979). PCPP was detected using a phosphothreonine antibody (Cell Signaling, Danvers, MA). D1 protein and its degradation fragments were detected using anti-D1 antibodies as described previously by Miyao (1994). To analyze oxidation of thylakoid membrane proteins, whole plants were dark-adapted overnight and leaf segments were illuminated at 1,500 µmol m^{-2}s^{-1} for up to 3 h. The oxidation of proteins in thylakoid membranes was detected using

the OxyBlot^{TM} Protein Oxidation Detection Kit (Millipore, Billerica, MA) following the manufacturer's instructions.

2.6. Coomassie staining

Coomassie staining was done to visualize the polypeptides composition in the SDS gel or in the membrane. Membrane or SDS gel was immerged in coomassie staining solution containing 0.25 g Coomassie Brilliant Blue R250, 45 ml methanol, 45 ml distilled water, 10 ml glacial acetic acid for 1 h at room temperature with gentle shaking. After that unspecific binding of coomassie dye was removed by destaining with destaining solution containing 45 ml methanol, 45 ml distilled water, 10 ml glacial acetic acid until the removal of unspecific binding of coomassie dye at room temperature.

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

BN-PAGE was performed using the procedure described by Reisinger and Eickacker (2006) with some modifications. Briefly, thylakoid membranes were isolated in grinding buffer containing 50 mM HEPES (pH 7.6), 0.3 M sorbitol, 10 mM sodium chloride, 5 mM magnesium chloride, 2 mM EDTA and 5 mM sodium ascorbate. The homogenate was filtered through three layers of miracloth (Calbiochem, CA). Pellets were then washed three times with washing buffer containing 50 mM HEPES (pH 7.6), 0.3 M sorbitol, 10 mM sodium chloride and 5 mM magnesium chloride. About 20 µg thylakoids were resuspended in 20% glycerol, 25 mM bis-tris, and 10 mM magnesium chloride

with 2% n-dodecyl-β-D-maltoside for 30 min at 4°C. The solubilized fraction was loaded onto a 5-13.5 % BN-gel.

2.8. Two Dimensional polyacrylamide gel electrophoreses (2D-PAGE)

After the native BN-PAGE electrophoresis, each lane of the gel was excised and incubated for 30 min at room temperature in a denaturing buffer containing 125 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 2% beta-mercaptoethanol, and 10% glycerol. The denatured gel stripwas loaded directly onto a 5% stacking gel for 2D-SDS-PAGE. The subunits of chlorophyll protein complexes were resolved on a 12% resolving gel. After electrophoreses at 80V for 2 h, proteins were transferred into the membrane. The membrane was incubated at 5% skim milk solution for 1 h. Finally, the membrane was incubated with D1 antibody to detect D1 protein.

2.9. Isolation of LHCII, PSI and PSII-LHCII supercomplex by sucrose density gradient ultracentrifugation (SDGU)

LHCII, PSI and PSII-LHCII supercomplex was isolated by using sucrose density gradient ultracentrifugation according to Tokutsu et al. (2012). About 400 µg thylakoid membranes were used to isolate different CP complexes. Each complex of isolated SDGU samples was collected then chlorophyll *a/b* ratio and absorption spectra were measured at room temperature by using Nano Drop ND-1000 spectrophotometer. In addition, the composition of LHCII, PSI and PSII-LHCII supercomplex of SDGU was further confirmed by BN-PAGE,

Immunoblotting with respective antibody and visualized by coomassie staining of the SDS gel.

2.10. Measurement of state transition

State transitions were measured according to Lunde et al. (2000) with some modifications using a pulse-amplitude-modulated fluorometer (PAM101/102/103, Walz Effeltrich, Germany). Briefly, dark adapted leaf discs were exposed to saturating white light for two seconds to determine Fm, and subsequently illuminated for 15 min with 50 µmol m^{-2}s^{-1} blue light (BL) to create state 2. The far-red light (FRL) was turned on to create state 1, and after 15 minutes, maximal fluorescence in state 1 (Fm1) was determined by giving the tissues a two second saturating white light pulse. The FRL was then switched off, the fluorescence was recorded with BL for 15 min, and the maximum fluorescence yield in state 2 (Fm2) was then determined by giving a two second saturating white light pulse. The relative change in maximal fluorescence was calculated as $\Delta F = (Fm1 - Fm2) / Fm2 \times 100$ according to Bellafiore et al. (2005).

2.11. Measurement of PSI activity

The redox state of P700 was determined with a pulse-amplitude-modulated fluorometer (PAM101/102/103, Walz Effeltrich, Germany) in the reflectance mode as described in Kim et al. (2005). The device was equipped with a dual-wavelength (810/870 nm) emitter-detector unit (ED-P700DW) consisting of a LED-driver unit and an emitter-detector unit (Walz). The PSI activity was expressed as the relative amount of far red light-induced P700$^+$ in

the leaf segments ($\Delta A810/A810$) after 5 min of pre-illumination with 120 µmol $m^{-2}s^{-1}$ actinic white light (Klughammer and Schreiber, 1994; Ivanov et al., 1998; Kim et al., 2005). A810 is the absorbance signal after the application of saturating far red light, and $\Delta A810$ is the saturating light-induced change in the absorbance signal during illumination with actinic light.

3. RESULTS

3.1. Phenotypic analysis of *osstn8* mutant plants

For rice plants cultivated in paddy fields as well as in the greenhouse, the growth rates of *osstn8* mutants were slower than WT, and the growth retardation resulted in a flowering delay of about 7-10 days (Fig. 7A, B, C). The mutant leaves were dark-green in color than WT, and the chlorophyll *a/b* ratio was 3.2 in WT and 3.0 in *osstn8* mutant leaves. The total chlorophyll content in the leaves was similar in WT and *osstn8* (Table 1). In *osstn8* mutant, the number of panicle was almost the same as compared with WT, and the length of panicle was similar to WT. However, total yield was less in *osstn8* mutant as compared to WT (Fig. 8A, B, C, D).

3.2. PSII core protein phosphorylation was significantly repressed in *osstn8* mutant leaves

Because Arabidopsis STN8 is required for PCPP (Bonardi et al., 2005; Vainonen et al., 2005), the endogenous phosphorylation of thylakoid membrane proteins in WT and *osstn8* mutant leaves was analyzed by immunoblotting using a phosphothreonine-specific antibody. Before analyzing the phosphorylation of thylakoid protein the gene expression was checked in the transgenic Comp-1 plants carrying a wild copy of *OsSTN8* gene (Fig. 9). In dark adapted WT leaves the phosphorylation of D1 and D2 was negligible, but phosphorylation of CP43 was shown at detectable levels (Fig. 10A, B). By
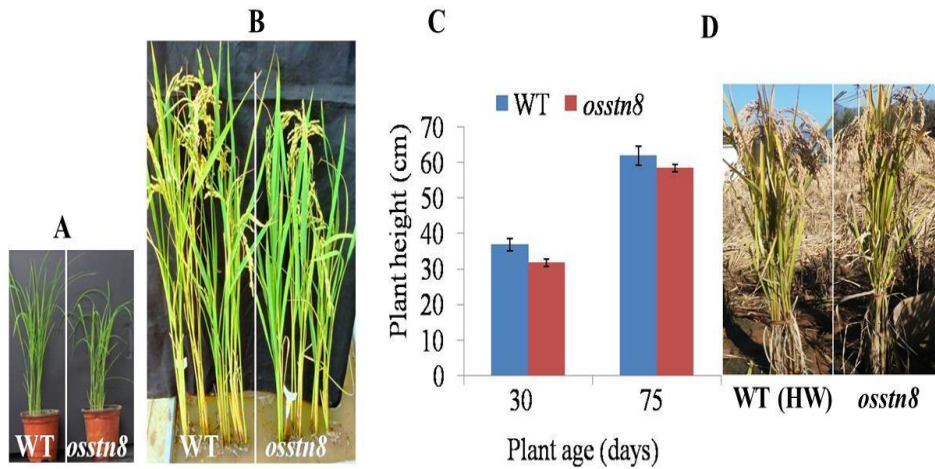
Figure 7. Phenotype of WT and *osstn8* mutants of rice. (**A**) WT and *osstn8* mutant plants grown in the green house at the age of one month old, (**B**) WT and *osstn8* mutant plants in the green house at the age of seed maturation, (**C**) Comparison of heights between WT and *osstn8* plants measured at the age of 30 and 75 days. Each value represents the mean of fifteen plants with the standard deviation indicated and (**D**) WT and *osstn8* plants grown in paddy field at the time of seed harvesting.

Table 1. Total chlorophyll concentration in leaves of WT and *osstn8* mutant.
The total chlorophyll content was estimated according to the method of Porra et al. (1989) in 80% acetone. All values are the average of three independent biological repeats given in µg chlorophyll/cm^{-2} with the standard deviation indicated ($\pm$).

	HW	*ossstn8*
Total chlorophyll (a+b)	34.21± 3.66	34.1± 2.07
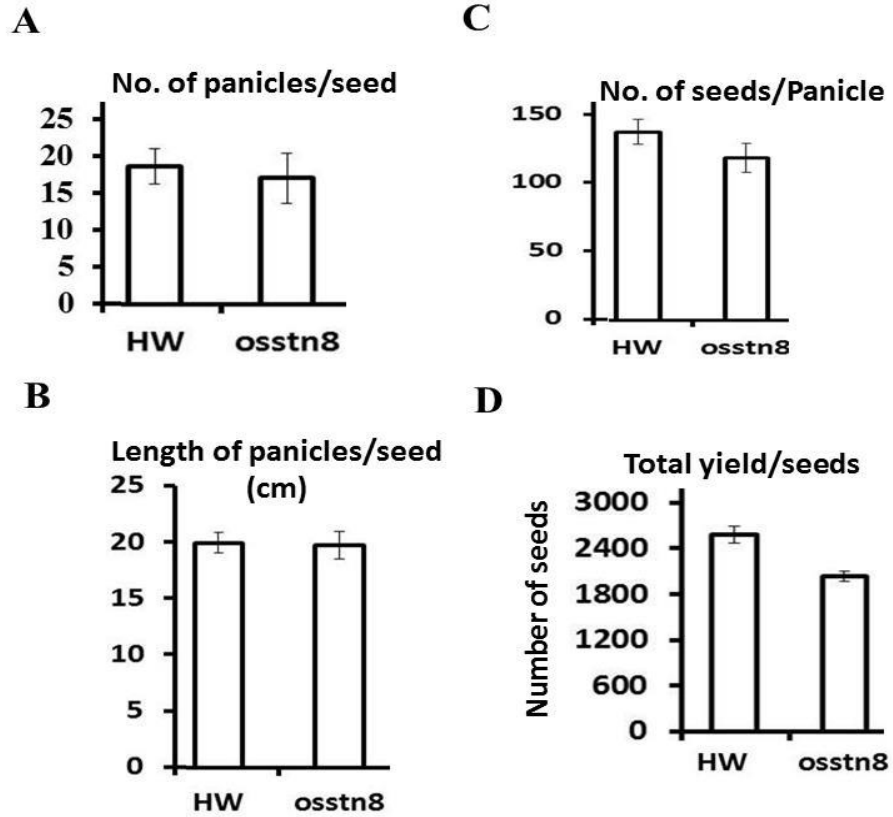
Figure 8. Phenotypic analysis of WT and *osstn8* mutants of rice. (**A**) Number of panicle per seed, (**B**) length of panicle per seeds, (**C**) Number of seeds per panicle and (**D**) Total yield of plants was calculated from several panicle obtained from one seed that was grown. Each data point represents the mean of ten plants with the standard deviation indicated.

illumination of either low light (LL) at 200 µmol m^{-2}s^{-1} or HL illumination at 1,500 µmol m^{-2}s^{-1} for 2 h, the phosphorylation of the reaction center proteins, D1 and D2, was induced strongly, but the intensity of CP43 was not changed significantly. In *osstn8* plants, phosphorylation of all PSII core proteins was significantly repressed. Phosphorylation of LHCII was also induced under LL and became negligible under HL, but it was not significantly repressed by the mutation of *OsSTN8* (Fig. 10A, B). The extent of phosphorylation of PSII core proteins and LHCII in the transgenic Comp-1 plants carrying a wild copy of *OsSTN8* gene (Fig. 9) was comparable to that of WT (Fig. 10A, B), suggesting that the specific inhibition of PCPP in *osstn8* mutants was due to the mutation of *OsSTN8*. Taken together, these datas indicate that OsSTN8 kinase is responsible for PCPP, but not for the phosphorylation of LHCII proteins, and therefore the state transition, in the monocot rice plant.

3.3. Mutant leaves were more susceptible to HL and light chilling

Because *osstn8* mutants showed growth retardation, its susceptibilities to HL and light chilling were compared with WT. Under GL, the Fv/Fm values of *osstn8* mutant leaves were found to be very similar to those of WT leaves (Fig. approximately 0.8, 11A, B), indicating that the mutant leaves had remained healthy. However, *osstn8* mutant leaves were found to be more susceptible to HL illumination at 2,000 µmol m^{-2}s^{-1} as well as light chilling treatment than WT leaves (Fig. 11A, B).

To test whether the higher susceptibility to photoinhibition in the mutant leaves was due to accelerated damage to PSII or to impaired repair of photodamaged PSII; the plants were illuminated in the presence of lincomycin, which blocks PSII repair by inhibiting chloroplast protein synthesis. The
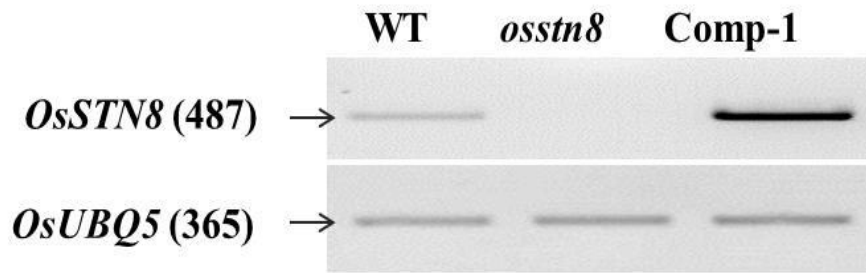
Figure 9. Complementation test of *osstn8* mutant of rice. RT-PCR of WT, *osstn8* mutant and *osstn8* complemented (Comp-1) plants using gene specific primers. *OsUBQ5* was used as internal control. Transgenic complement plant was confirmed by checking the gene expression of STN8 kinase.
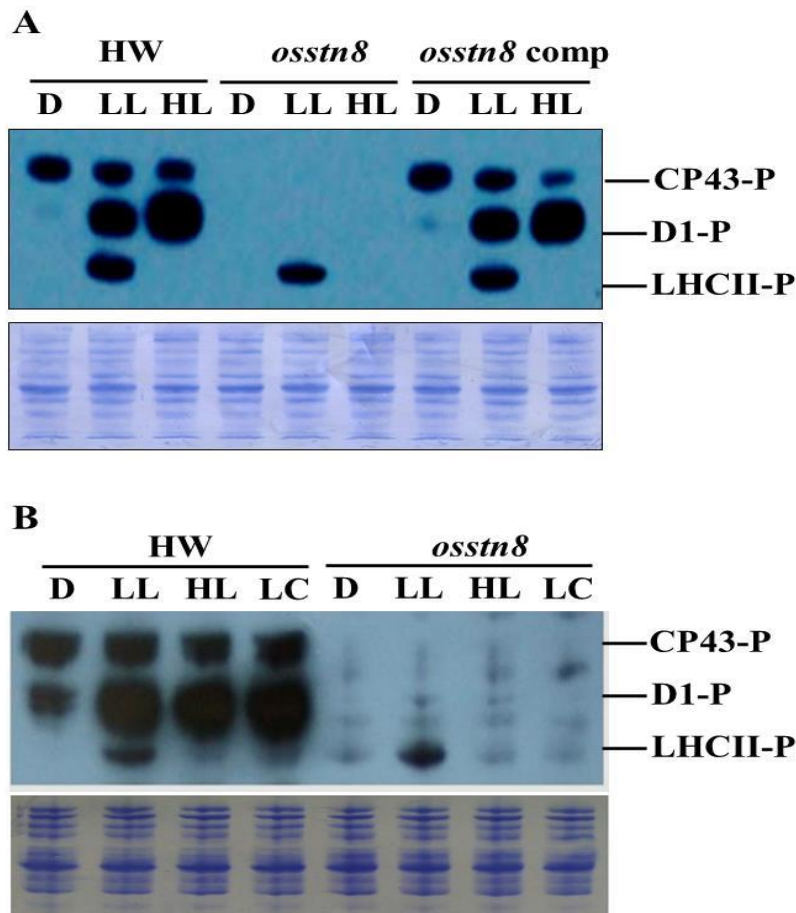
Figure 10. Analysis of phosphorylation of thylakoid membrane protein by immunoblotting with phosphothreonine antibody. (**A**) Immunoblotting analysis of phosphoproteins in WT, *osstn8* and *osstn8* complement plant (Comp-1). (**B**) WT and *osstn8* with including light chilling treatment. The thylakoid membranes containing 2 µg chlorophyll was used in each lane. The SDS-PAGE after commassie blue stainingof the gel is shown in the bottom of each figure. D = dark adapted leaves sample, LL = low light at 200 µmol $m^{-2}s^{-1}$ for 2 h, HL = high light at 1,500 µmol $m^{-2}s^{-1}$ for 2 h and LC = light chilling at 200 µmol $m^{-2}s^{-1}$ for 12 h.

40

addition of lincomycin induced a more pronounced degree of PSII inactivation and the Fv/Fm decreased at similar rates between WT and *osstn8* mutant leaves (Fig. 11B). The results obtained in the presence of lincomycin suggest that the rate of PSII inactivation in the mutant leaves under HL illumination does not differ from that of WT leaves. However, in the absence of lincomycin, the decrease in the Fv/Fm in the mutants was more rapid than that in WT (Fig. 11B), suggesting that the PSII repair system was significantly impaired in the *osstn8* leaves. In both plant types upon treatment with lincomycin, the decreased Fv/Fm did not recover under dim light (Fig. 11C), indicating that lincomycin infiltration was sufficient to block the *de-novo* synthesis of D1 protein almost completely, which was also found to be the case in the samples illuminated with HL at different intensities (Nath et al., 2013b).

3.4. State transition was similar between WT and *osstn8*

State transition was detected as differential changes in fluorescence from PSII when leaves are exposed alternately to light favouring PSII and light favouring PSI. The relative change in maximal fluorescence during state transition, ΔF was 6.25 % in WT and 6.45 % in *osstn8* mutant as shown in Fig. 12A and B. Maximal fluorescences were measured between Fm1 and Fm2' at the interval of 2 min and the fluorescence pattern in the mutant was similar to that in WT (Fig. 12C, D).

3.5. D1 protein degradation was suppressed in *osstn8* mutant leaves
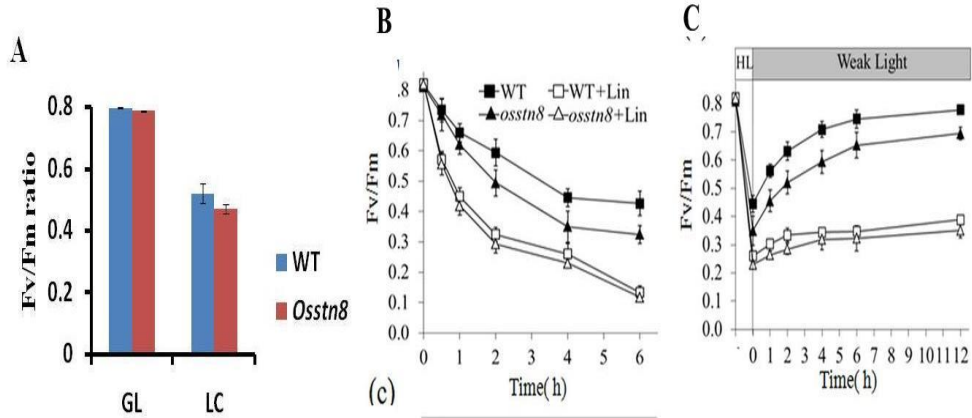
Figure 11. PSII photoinactivation of PSII during HL illumination and light chilling treatment. Fv/Fm ratio was calculated after high light and light chilling treatment in WT and *osstn8* mutant leaf samples. (**A**) Fv/Fm ratio of WT and *osstn8* mutant after high light and light chilling treatment. GL = low growth light at 200 µmol m^{-2}s^{-1} for 2 h and LC = light chilling at 4-5°C with 200 µmol m^{-2}s^{-1} for 15 h. (**B**) Decline of photochemical efficiency of PSII or Fv/Fm during photoinhibition under 2,000 µmol m^{-2}s^{-1} light intensity in the presence/absence of the plastid protein synthesis inhibitor lincomycin (Lin) for up to 6 h and (**C**) subsequent recovery for 4 h of photoinhibited samples under dim light intensity of 20 µmol m^{-2}s^{-1} for up to 12 h. Values are the means ± SD of five replicates. Figure B and C adopted from Nath et al. (2013b).
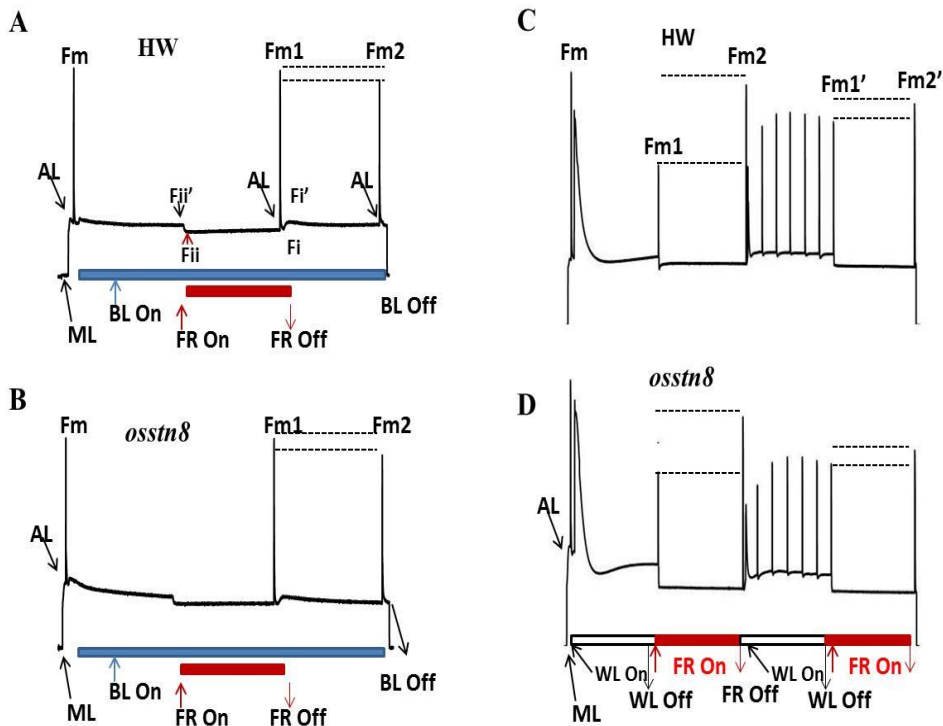
Figure 12. Measurement of state transition. (**A**) State transition in WT and (**B**) State transition in *osstn8* mutant.Uphead blue arrow: turn on blue light, uphead red arrows: turn on far-red light, downhead red arrow: turn off far red light, downhead blue arrow: turn off blue light. ML = measuring light, BL = blue light, FR = far red light, Fm, Fm1 and Fm2 was obtained by applying the saturated pulse at 4,000 µmol m^{-2}s^{-1} for 0.5 seconds. Similarely, maximal fluorescences were measured between Fm1 and Fm2' at the interval of 2 min. (**C**) Maximal fluorescences in WT and (**D**) in *osstn8*.

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To examine whether the susceptibility of *osstn8* mutants to HL illumination was related to the preferred degradation pathway for D1 protein, immunoblotting experiments for D1 protein and its degradation products were performed. This analysis revealed that the level of D1 protein decreased both in WT and *osstn8* complement leaves after HL illumination in the absence of lincomycin, but the extent of the decrease was less in *osstn8* mutants compared with WT or Comp-1 lines (Fig. 13A). In the presence of lincomycin, where the *de novo* synthesis of D1 protein is blocked, the difference in the level of D1 protein between the mutant and WT (or Comp-1) leaves after HL illumination became greater, suggesting that the degradation of photodamaged D1 protein was repressed in the mutant leaves. This result was verified by comparing the band intensities of degradation products of D1 protein. The level of D1 fragment accumulation was less in *osstn8* mutant leaves compared with WT (or Comp-1) leaves, particularly in the absence of lincomycin (Fig. 13A). These results were verified again using an antibody raised against the DE loop of D1 protein, suggesting that the degradation process for D1 was repressed as a consequence defected PCPP by the mutation in the *OsSTN8* gene (Fig. 13A). On the contrary, degradation of D1 protein was similar between WT and *osstn7* (Fig. 13B). The result suggests that PCPP but not LHCII phosphorylation is essential for PSII repair cycle in rice plant.

3.6. The photoinactivation of photosystem I (PSI) was similar between WT and *osstn8* mutants

Because superoxide is believed to be produced by PSI in plants under stress conditions (Asada, 1999; Sonoike, 2006), PSI activity as the relative amount of far red light-induced P700$^+$ (by $\Delta A810/A810$) has been measured in
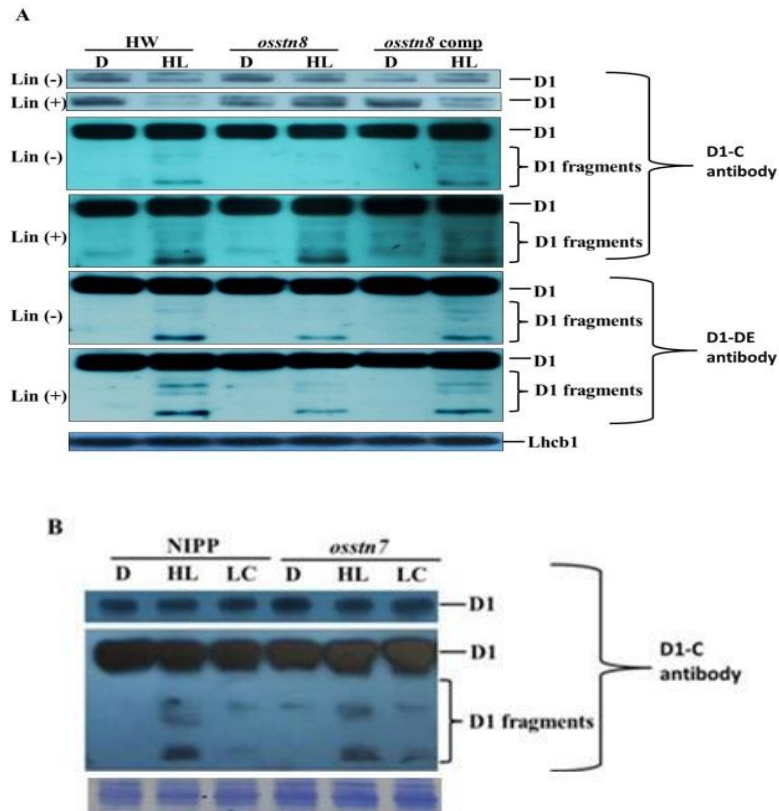
Figure 13. Immunoblotting of thylakoid membrane protein in WT, *osstn7*, *osstn8* and *osstn8* complement plant by using D1 antibody. (**A**) Immunoblotting analysis of D1 protein and its degradation in WT, *osstn8* and *osstn8* complement (Comp-1) against D1 antibodies before and after HL treatment in the presence and absence of lincomycin (+ Lin and - Lin). Lhcb1 was used as a loading control, (**B**) Immunoblotting analysis of D1 protein and its degradation in WT and *osstn7* plants against D1 antibodies before and after HL treatment. Aliquot of thylakoid membranes containing 0.5 µg chlorophyll was loaded in each lane. Coomassie staining of membrane shows the loading control. in the bottom panal. D = Dark adopted leaf sample and HL = high light illuminated leaf sample at 1,500 µmol m^{-2}s^{-1} for 2 h respectively, LC = light chilling at 4°C for 15 h.

the leaves of WT and *osstn8* mutant plants. The PSI activity in the mutant leaf segments was found to be similar to that of WT (Table 2). After HL illumination at 1,500 µmol m^{-2}s^{-1} for 1 h, the PSI activity decreased by 41.6% and 39.1% in WT and *osstn8* mutants, respectively, compared with their controls, indicating that the photoinactivation of PSI in *osstn8* mutants was not more severe than WT.

3.7. The mobilization of PSII-LHCII supercomplex was suppressed in the *osstn8* mutant

In Arabidopsis *stn7/stn8* double mutants, the PSII-LHCII supercomplex was not likely to be properly disassembled to enable the mobilization of damaged proteins for degradation during HL illumination (Tikkanen et al., 2008). In BN-PAGE experiment, the mobilization of PSII supercomplex was observed in WT plants during HL illumination as well as light chilling treatment, and this process was significantly blocked in *osstn8* mutants, a result which was also confirmed in the SDGU experiment (Fig. 14A, B). The mobilization of PSII supercomplex was observed in WT plants during HL illumination, and this process was significantly blocked in *osstn8* mutant (Fig 14A, B). This result suggests that defects in PCPP in *osstn8* mutant leaves block the disassembly of PSII-LHCII supercomplex.

To check whether the damaged PSII centers in the mutant accumulate and degraded as PSII monomers, the 2D gels were run by using BN-gel of WT and *osstn8* mutant. In WT, more PSII complexes were monomerized and D1 degradation was faster, whereas in the *osstn8* mutant D1 degradation was slower and most of the D1 was still in PSII dimer and even in PSII supercomplex (Fig. 15). This result suggests that mutant accumulated damaged PSII reaction center during HL illumination. The composition of each complex isolated by SDGU was

Table 2. Photoinactivation of PSI in leaves of WT and *osstn8* mutant. PSI activity was measured before and after HL illumination at room temperature.

	Dark		HL	
	HW	***ossstn8***	**HW**	***ossstn8***
ΔA810/A810	0.67 ± 0.07	0.69 ± 0.05	0.39 ± 0.06	0.42 ± 0.13

SDGU was further confirmed by BN-PAGE, immunoblotting with respective antibodies and visualization by Coomassie staining of the SDS gel. (Fig. 16A, B, C).

Mobilization of PSII supercomplex was further examined, using single mutants of Arabidopsis *Atstn7*, *Atstn8* and *AtCP29*-KO. The result showed that PSII supercomplex were mobilized both in *stn7* and *stn8* mutant (Fig. 17A). However, there was not complete inhibition of phosphorylation of PSII core and LHCII proteins in each mutant of Arabidopsis *stn7* and *stn8* mutants (Fig. 17B). But mutation of CP29 protein in Arabidopsis shows less amount of PSII supercomplex (Fig. 17A).

3.8. Protease was essential for mobilization and degradation of PSII supercomplex

To investigate any possible role of protease in the mobilization of PSII supercomplex during high light illumination, protease inhibitor infiltrated leaf samples were illuminated with HL and subjected to BN-PAGE as well as immunoblotting analysis. Two different protease inhibitors such as phenyl methyl sulfonyl fluoride (PMFS) and protease inhibitor cocktail (Cocktail, purchased from Sigma) were infiltrated in leaf sample. Interestingly, in the presence of protease of protease inhibitors, PSII supercomplex was not mobilized during HL illumination and D1 degradation was also blocked (Fig. 18A, B). But, treatment of protease inhibitors had no influence on the phosphorylation of PSII proteins (Fig. 18C). Although, infiltration of cocktail showed more effective than PMSF. This result indicates that PSII core protein phosphorylation was not enough to mobilized PSII supercomplex.
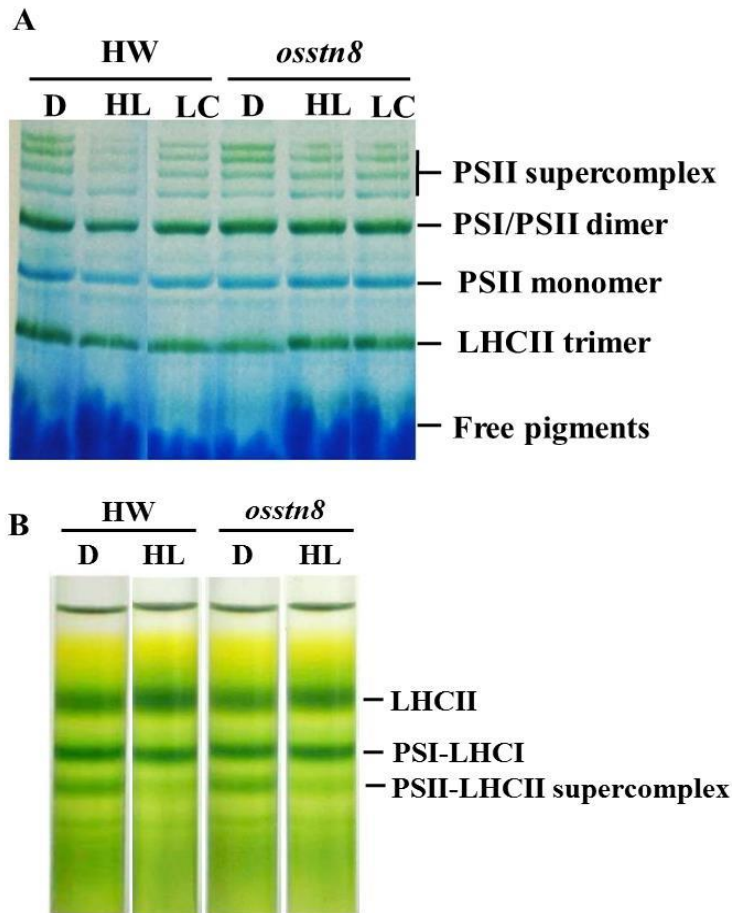
Figure 14. Separation of PSII-LHCII supercomplex by BN-PAGE and sucrose density gradient ultracentrifugation. About 10 µg thylakoids from each plant's leaves were solubilized in n-dodycyl β-D-maltoside, and separated by 5-13.5 % BN-PAGE and different chloroplast protein (CP) complexes were separated. (**A**) BN-PAGE of rice WT and *osstn8* mutant, (**B**) Isolation of LHCII, PSI and PSII-LHCII supercomplex in WT and *osstn8* mutant by using sucrose density gradient ultracentrifugation. D = dark adapted leaves sample, HL = high light at 1,500 µmol m^{-2}s^{-1} for 2 h and LC = light chilling at 200 µmol m^{-2}s^{-1} for 12h.

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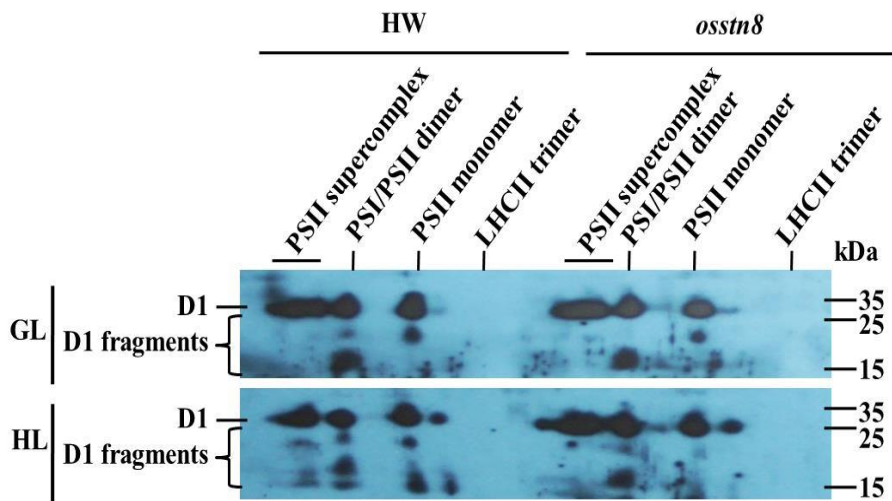
Figure 15. Analysis of mobilization of damaged D1 protein by using two dimensional SDS gel electrophoreses. BN-PAGE was performed in WT and *osstn8* mutant as shown in Figure 14A. Two dimensional of BN-PAGE was performed for immunoblotting with D1 antibody. D = dark adapted leaves sample, HL = high light at 1,500 µmol m^{-2}s^{-1} for 2 h.
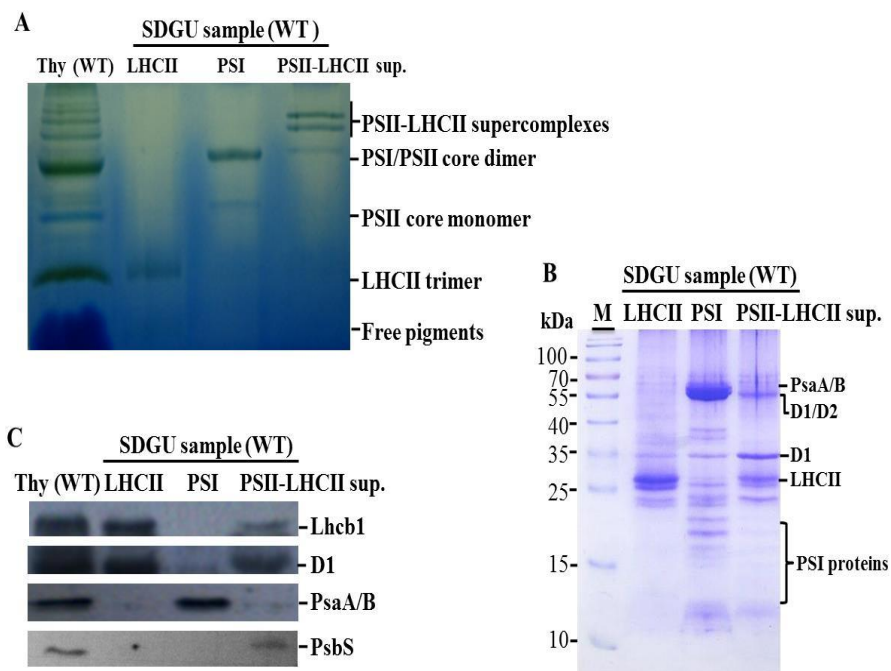
Figure 16. Confirmation of sucrose gradient samples of LHCII, PSI and PSII-LHCII supercomplex from WT plants. Isolated CP complexes by using Sucrose density gradient ultracenterfugation in Figure 14B was confirmed by using several methods. (**A**) BN-PAGE analysis of each complexeobtained by sucrose gradient from WT samples. Each complexes obtained by sucrose gradient were separated by 5-12.5% BN-PAGE. Isolated thylakoidsfrom WT (Thy WT) were used as a positive control. Each complex resolved usinga sucrose density gradient is indicated above the gel, (**B**) Protein profile of LHCII, PSI and PSII-LHCII supercomplex of WT sample of sucrose gradient where kDa represents the protein marker size in kilodalton, (**C**) Immunoblotting detection of major proteins of the LHCII, PSI and PSII-LHCII supercomplex obtained by SDGU. Lhcb1, D1 and PsbS antibodies were used as marker proteins for LHCII and PSII-LHCII supercomplex and PsaA/B antibody was used as a marker protein for PSI complex.
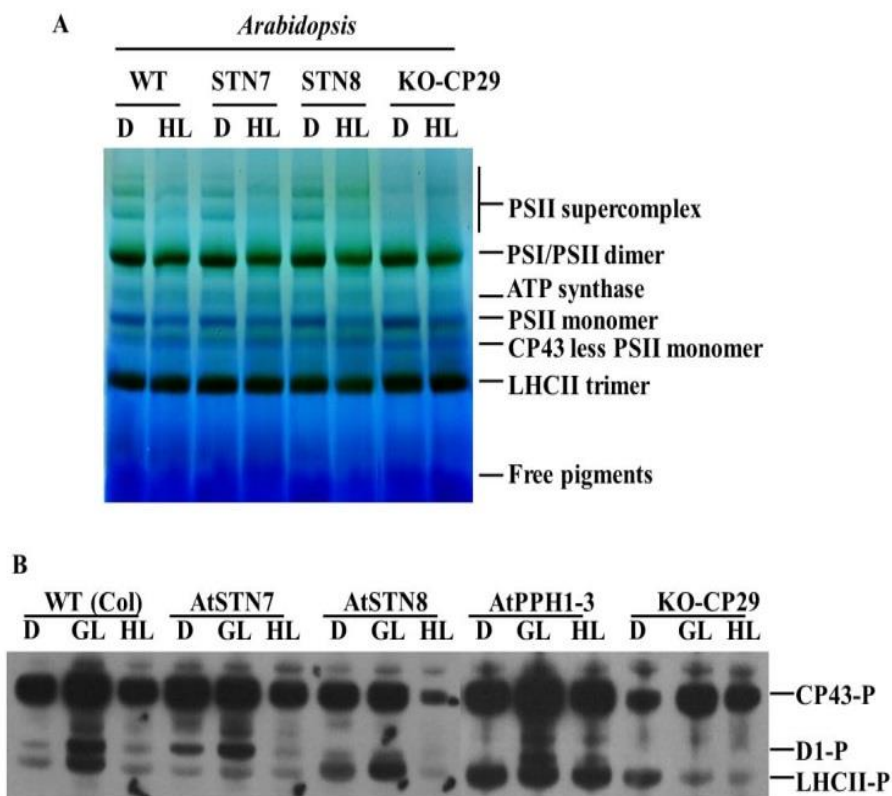
Figure 17. Separation of PSII-LHCII supercomplex by BN-PAGE and phosphorylation of PSII-LHCII proteins in Arabidopsis. (**A**) About 10 µg thylakoids from WT, *stn7*, *stn8* and KO-CP29 (CP29 knock out mutant) plant's leaves were solubilized in n-dodycyl β-D-maltoside, and separated by 5-13.5 % BN-PAGE and different chloroplast protein (CP) complexes were separated. (**B**) Immunoblotting analysis of phosphoproteins in WT, *stn7*, *stn8*, pph1-3, and *KO-CP29* mutants. The thylakoid membranes containing 2 µg chlorophyll was used in each lane. Immunoblotting was conducted by using phosphothreonine antibody.
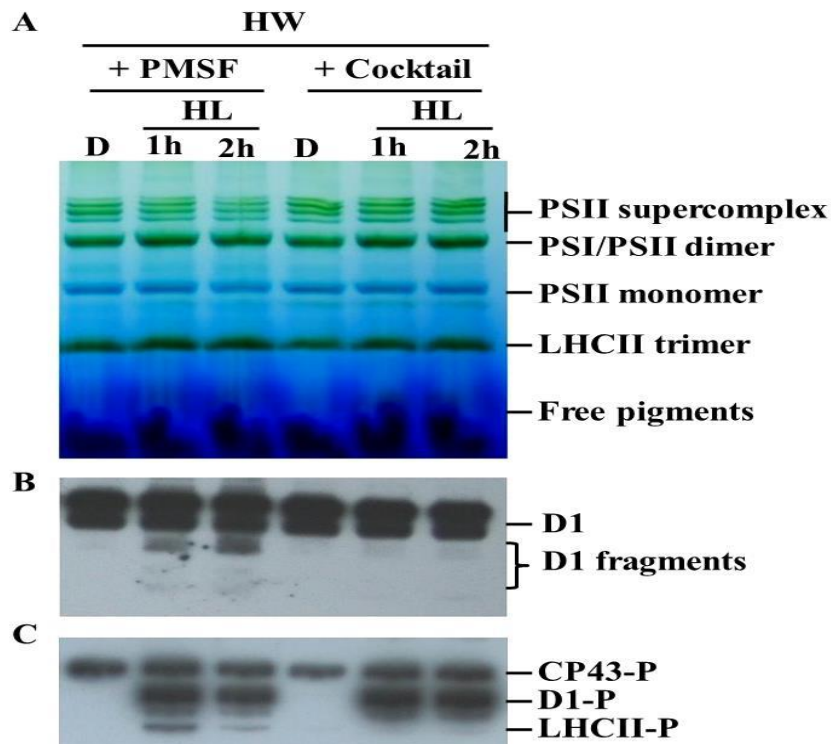
Figure 18. Analysis of mobilization of PSII supercomplex, degradation of D1 protein and phosphorylation of PSII-LHCII proteins in protease inhibitor infiltrated leaf sample of WT rice. Leaf segments were infiltrated with phenyl methyl sulfonyl fluoride (PMSF) and protease inhibitor cock tail (Cocktail) and applied to high light illumination. Thylakoid membranes were isolated and equal amount of thylakoid membranes were used. (**A**) About 10 µg thylakoids from each plant's leaves were solubilized in n-dodycyl β-D-maltoside, and separated by 5-13.5 % BN-PAGE and different chloroplast protein (CP) complexes were separated, (**B**) Immunoblotting analysis of D1 protein and its degradation in WT, (**C**) Immunoblotting analysis of phosphoproteins in WT. Aliquot of thylakoid membranes that containing 0.5 µg chlorophyll was loaded in each lane for immunoblotting assay. D = dark adapted leaves sample, HL = high light at 1,500 µmol m^{-2}s^{-1} for 1 and 2 h.

3.9. The oxidation of thylakoid proteins was elevated in *osstn8* plants

The consequence of mutation of STN8 kinase was observed in terms of oxidative stress in plants. When the leaves were illuminated with HL light, the oxidation of thylakoid proteins was found more pronounced in mutant as compared to WT. Higher production of ROS in the mutant leaves was observed when comparing the oxidation of thylakoid proteins between WT and *osstn8* mutant leaves during HL illumination at an intensity of 1,500 µmol m^{-2}s^{-1} for up to 3 h. In *osstn8* plants PSII core proteins were found to be carbonylated more rapidly than in WT (Fig. 19). These results again indicate that higher levels of ROS are produced in *osstn8* plants than in WT, particularly from PSII complexes.
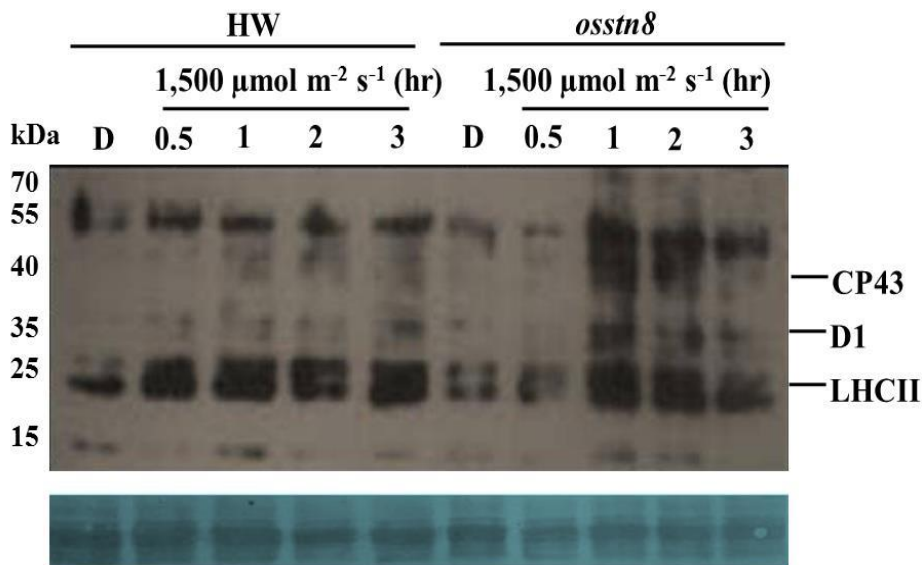
Figure 19. Analysis of reactive oxygen production in thylakoid membranes of WT and *osstn8* mutants by using Oxyblot assay. Immunoblotting analysis of oxidation of thylakoid membranes proteins in WT and *osstn8* mutant plants before and after high light at 1,500 µmol $m^{-2}s^{-1}$ for 30 min, 1, 2 and 3 h illumination. The thylakoid membranes containing 5 µg chlorophylls were loaded in each lane and kDa represents the protein marker size in kilodaltons. Botton panel shows the SDS-PAGE after commassie blue staining showing major LHCII proteins as a loading control.

4. DISCUSSION

The results obtained in this study of a monocot model rice plant strongly support the classical view of the PSII repair cycle (Aro et al., 1993), which was challenged by Bonardi et al. (2005) on the basis of studies carried out in *stn8* single and *stn7/stn8* double mutants of Arabidopsis suggested that protein phosphorylation by STN8 kinase was not essential for PSII repair.

In rice, the mutation of *OsSTN8* alone was found to be sufficient to significantly suppress PCPP (Fig. 10), in contrast to Arabidopsis *stn8* single mutants in which PCPP was little affected (Bonardi et al., 2005; Tikkanen et al., 2008; Fristedt et al., 2009). Defects in PCPP in *osstn8* mutants were also enough to suppress the mobilization of PSII supercomplex under HL illumination as shown by BN-PAGE and SDGU experiments (Fig. 14A, B). Because the damaged PSII was mostly unable to be mobilized for degradation in *osstn8* mutants, the degradation of D1 protein was largely suppressed in *osstn8* plants (Fig. 13A). Lincomycin infiltration experiments (Fig. 13A) further suggested that the rate of photodamage in PSII in *osstn8* mutants was similar to WT, but that the repair process was blocked in the mutants. Hence, PSII of the *osstn8* mutants was highly susceptible to HL illumination (Fig. 11A, B). In the case of Arabidopsis *stn7/stn8* mutants, PSII inactivation under HL illumination was not found previously to be significant (Bonardi et al., 2005) or became noticeable following prolonged illumination (Tikkanen et al., 2008).

Although the STN8 kinase is specific to PCPP in Arabidopsis, STN7 seems to be also involved in this process, because significant suppression of both PCPP and mobilization of PSII supercomplex was found to be only possible in

the *stn7/stn8* double mutant of Arabidopsis (Bellafiore et al., 2005; Bonardi et al., 2005; Vainonen et al., 2005; Tikkanen et al., 2008; Frietedt et al., 2009). In contrast, the obtained results in rice reveal that the mutation in *OsSTN8* alone was sufficient to produce all of the phenotypes observed in the double mutants of Arabidopsis, which suggests that the overlap of the function or cross-talk of STN7 and STN8 for PCPP is much less in the monocot rice plant, compared with dicot Arabidopsis. In addition, the growth of *osstn8* mutants was slightly retarded (Fig. 7), and the time of flowering and seed harvest time were delayed as compared to WT (Fig 7B).

In contrast, the phosphorylation of LHCII protein is specific to STN7 in Arabidopsis (Bellafiore et al., 2005; Tikkanen et al., 2008), and the process was not influenced significantly by the mutation of *OsSTN8* in rice (Fig. 10A, B). The phosphorylation of CP43 was not found to be light-induced unlike other PSII core proteins including D1 and D2 (Fig. 10A, B), suggesting that the phosphorylation of CP43 is differently regulated from that of D1 and D2, although all of these PSII core proteins are phosphorylated by STN8 in rice. The differential regulation of CP43 phosphorylation was also reported previously in Arabidopsis (Bonardi et al., 2005; Vainonen et al., 2005; Tikkanen et al., 2008). CP43 phosphorylation was reported to be important for PSII supercomplex mobilization in Arabidopsis (Dietzel et al., 2011). Consistently, similar result has obtained in the rice plant in the research (Fig. 10A, B).

In the PSII repair cycle, photodamaged and phosphorylated PSII moves from the granal to stromal areas for subsequent degradation of the D1 protein, and then eventually replaced by newly synthesized D1 in the newly assembled PSII complex (Aro et al., 1993; Tikkanen et al., 2008). In this linear process, the PSII repair cycle can be blocked either at the phosphorylation step or degradation step.

It was reorted earlier that when the degradation step was blocked by the mutation of the responsible proteases, the repair cycle seemed to stop working, as shown in FtsH knock-out mutants (Bailey et al., 2002) and in *deg1* mutants (Kapri-Pardes et al., 2007). In these mutants, the degradation of D1 was significantly suppressed, indicating that the degradation steps involving these two proteases are unique to the PSII repair cycle. However, earlier work showed that the inhibition of DEG5 or DEG8 protease did not seem to block the repair cycle completely, but a double mutation of these two proteases might do so (Sun et al., 2007). Similarly, partial inactivation of PSII was also observed in the *deg5* or *deg7* mutant, and the degree of PSII photoinactivation was more severe in the corresponding double mutants (Sun et al., 2010a).

In this study, the blockade of the degradation step in the PSII repair cycle in *osstn8* mutants was found to be partial based on the results of PSII photoinactivation experiments (Fig. 11A, B), In contrast to this, the phosphorylation step for the photodamaged D1 protein was significantly blocked in *osstn8* mutants (Fig. 10A). Moreever PSII supercomplex was not mobilized efficiently in *osstn8* mutants (Fig. 14A, B). Photodamaged D1 protein that is not mobilized and not phosphorylated may be degraded slowly by unknown housekeeping proteases, although the degradation of D1 protein after mobilization is mainly governed by FtsH and Deg proteases.

The current analyses of the oxidation of thylakoid proteins suggest that PSII core proteins are oxidized to a greater extent in *osstn8* mutants than in WT (Fig. 19). In Arabidopsis, a preferential oxidation of photosynthetic machineries was observed only in the *stn7/stn8* double mutants that lack complete PCPP (Tikkanen et al., 2008). Under stress, superoxide is believed to be produced mostly by PSI (Asada, 1999; Sonoike, 2006). However, the suppression of the far

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red light-induced $P700^+$ formation in the leaves was not more severe in the mutants compared with WT after HL illumination, and this was also the case in both plant types before HL illumination (Table 2). The present study suggests that the PSII supercomplex with damaged-but-not-phosphorylated PSII core proteins were unable to mobilize under HL illumination and are the possible site of superoxide production in *osstn8* mutants, but the underlying mechanism of this superoxide production remains to be further investigated.

Under growth light, *osstn8* mutants that had been grown in rice paddy field showed growth retardation, and also delayed flowering and seed harvesting times compared with WT. In contrast, no phenotype was reported in the Arabidopsis single *stn8* mutant (Bonardi et al., 2005; Tikkanen et al., 2008). Indeed growth retardation was observed only in the *stn7* and *stn7/stn8* double mutants in Arabidopsis (Bonardi et al., 2005; Frenkel et al., 2007; Tikkanen et al., 2008). These results suggest that the suppression of PCPP significantly influences the structure and function of plants under normal growth conditions. Grana stacking were reduced and the length of grana became longer in *osstn8* mutants (Nath et al., 2013b), as occurs in the *stn7/stn8* mutant of Arabidopsis (Fristedt et al., 2009). This macroscopic rearrangement of the entire network of thylakoid membranes with elongated grana might be due to the hindrance of lateral migration of photodamaged PSII complexes due to a deficiency of PCPP, in agreement with Fristedt et al. (2009). In conclusion, under HL illumination, PSII repair cycle involving PCPP and the migration of PSII supercomplex was blocked in the *osstn8* mutants, and for those reasons the *osstn8* mutant is considerd to show susceptibility to HL illumination, although the Arabidopsis *stn7/stn8* mutants became susceptible only after prolonged illumination (Tikkanen et al., 2008).

PART II

Production of Superoxide from Photosystem II-Light Harvesting Complex II Supercomplex in *OsSTN8* Knock-out Rice Mutants under Photoinhibitory Illumination

ABSTRACT

When phosphorylation of Photosystem (PS) II core proteins is blocked in STN8 knock-out mutants of rice (*Oryza sativa*) under photoinhibitory illumination, the mobilization of PSII supercomplex is prevented. Previously it has been proposed that more superoxide was produced from PSII in the mutant (Nath et al., 2013, Plant J., 76: 675-686). Here, the presented data clarify the type and site for the generation of reactive oxygen species (ROS). Using both histochemical and fluorescence probes, it has been observed that, when compared with wild-type (WT) rice leaves, levels of ROS, including superoxide and hydrogen peroxide, were increased when leaves from mutant plants were illuminated with excess light. However, singlet oxygen production was not enhanced under such conditions. When superoxide dismutase was inhibited, superoxide production was increased indicating that superoxide production is the initial event prior to hydrogen peroxide production. In thylakoids isolated from WT leaves, the kinase was active in the presence of ATP, and spectrophotometric analysis of nitroblue tetrazolium absorbance for superoxide confirmed that PSII-driven superoxide production was more in the mutant thylakoids than in the WT.

This contrast in levels of PSII-driven superoxide production between the mutants and the WT plants was confirmed by conducting protein oxidation assays of PSII particles from *osstn8* leaves under strong illumination. Those assays also demonstrated that PSII-LHCII supercomplex proteins were oxidized more in the mutant, thereby implying that PSII particles incur more damage even though that step in degradation is partially blocked. These results suggest that superoxide is the major form of ROS produced in the mutant, and that the damaged PSII in the supercomplex is the primary source of superoxide.

1. INTRODUCTION

Although plants convert light into chemical energy during photosynthesis, excessive irradiance levels can be harmful. PSII is vulnerable to high light (HL) illumination, which causes photoinhibition, a process determined by the balance between the rate of photodamage to PSII and its repair (Prasil et al., 1992). Photosynthetic organisms utilize a multi-step strategy, i.e., the PSII repair cycle, to prevent the accumulation of photo-damaged PSII proteins. Turnover of those damaged PSII core proteins, in particular D1 protein (Nath et al., 2013a), requires several highly organized stages: 1) reversible phosphorylation and de-phosphorylation of core proteins by STN8 kinase (Bonardi et al., 2005, Vainonen et al., 2005) and the PSII core phosphatase PBCP (Samol et al., 2012), respectively; 2) monomerization and disassembly of PSII complexes; 3) proteolytic degradation of damaged D1 protein; 4) de novo synthesis of D1 protein and its insertion into the partially disassembled PSII complex; and (5) reassembly of that PSII complex.

Kinases specific to the phosphorylation of LHCII and PSII core proteins are named STN7 and STN8, respectively (Bonardi et al., 2005; Vainonen et al., 2005). The availability of their single and double mutants in *Arabidopsis thaliana* (hereafter Arabidopsis) has made it easier to understand the regulatory mechanism for PSII core protein phosphorylation during the PSII repair cycle. However, the classic role of phosphorylating damaged D1 protein for PSII repair has been challenged by Bonardi et al., (2005) who characterized of the Arabidopsis *stn8* and *stn7*x*stn8* double mutant. Because the mutation of both kinase genes did not render PSII very sensitive to photoinhibition, the authors suggested that phosphorylation of the STN8-mediated PSII core protein is

perhaps not crucial for D1 protein turnover and PSII repair. However, it is shown that a single mutation of rice *STN8* is sufficient to produce most of the phenotypes observed in *stn7*x*stn8* double mutants of Arabidopsis, there by suggesting that the overlap of functioning or cross-talk between STN7 and STN8 is much less in the monocot model system of rice than in the model dicot Arabidopsis (Nath et al., 2013b). All characteristic features of the *osstn8* mutants seem to be a direct consequence of the suppression of PSII core protein phosphorylation. This supports the hypothesis that the essential role of STN8-mediated phosphorylation of PSII core proteins, in particular D1 protein, is as part of the PSII repair mechanism during photoinhibition.

Under both in vivo and in vitro photoinhibitory conditions, the *osstn8* mutants produce more reactive oxygen species (ROS) than wild-type (WT) plants (Nath et al., 2013b). Similarly, in Arabidopsis, the production of ROS, in particular the superoxide anion radical ($O_2^{.-}$) (hereafter superoxide) and hydrogen peroxide (H_2O_2), has been demonstrated in *var2* mutants, where the protein degradation stage in the PSII repair cycle is blocked due to the mutation of *FtsH* (Miura et al., 2010). The PSII-LHCII supercomplexes are highly abundant in the grana regions of the thylakoid membranes, and are mobilized to the granal margin, where damaged proteins are degraded by housekeeping chloroplast proteases such as FtsH and Deg (Puthiyaveetil et al., 2014; Yoshioka-Nishimura and Yamamoto, 2014). It has been previously reported that more superoxide seems to be produced from the PSII of *osstn8*, even though mobilization of PSII supercomplex was blocked (Nath et al., 2013b).

Because the generation site for each type of ROS depends upon the stress applied, it can be difficult to detect a particular ROS and locate its site of production (Zulfugarov et al., 2011, Zulfugarov et al., 2014; Foyer and Shigeoka

2011; Schmitt et al., 2014), even though the main source of ROS in the chloroplasts is the electron transport chain (Foyer and Noctor, 1999; Laloi and Apel, 2004; Mittler et al., 2004; Foyer and Shigeoka, 2011). Therefore, in this study, several biochemical, biophysical, and molecular biology approaches has been applied to clarify individual types of ROS and their sites of production in *osstn8* mutants. In this dissertation, it is suggested that the damaged PSII in PSII-LHCII supercomplex is possibly the main site of superoxide production in response to excess levels of light.

2. MATERIALS AND METHODS

2.1. Plant materials and growth conditions

From a T-DNA inserted mutant pool, *OsSTN8* knock-out mutant *osstn8* was used as described in Nath et al. (2013b). Wild-type (WT) rice (*Oryza sativa* cultivar 'Hwayoung') and *osstn8* lines were used in the experiments. One-month-old plants were used to conduct the experiments that were grown in a glasshouse at $30^{\circ}C/26^{\circ}C$ with a 16/8 h day/night photoperiod. The source of growth light in glasshouse was sun light that changes from 0 to 1,200 μmol m^{-2}s^{-1} in whole year in Busan, Republic of Korea. Before performing the experiments, plants were kept in rice growth chamber with constant light intensity at 100 μmol m^{-2}s^{-1} for 4-6 h for acclimation. Leaf segments were prepared and float on water then leaves were dark adapted for 6 h before light treatment.

2.2. Photoinhibitory treatment and measurement of chlorophyll concentrations

Prior to photoinhibitory illumination (i.e., high-light treatment = HL), plants were acclimated in a growth chamber and exposed for 4 to 6 h to a constant 100 μmol m^{-2}s^{-1}. Afterward, 2- to 3-cm-long leaf segments were excised, floated on water, and dark-adapted for 6 h. For HL, the segments were then illuminated for 2 h with a metal halide lamp (1,500 μmol m^{-2}s^{-1}) equipped with a water bath to reduce the temperature. Samples were frozen in liquid nitrogen for further experiments. Chlorophyll concentrations in the isolated thylakoids were measured after extracting the pigments with 80% acetone (Porra et al., 1989).

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

For separating the chlorophyll protein (CP) complexes, BN-PAGE was performed according to a standard method (Reisinger and Eichacker, 2006), but with some modifications. Briefly, the thylakoid membranes were isolated in a grinding buffer containing 50 mM HEPES (pH 7.6), 0.3 M sorbitol, 10 mM sodium chloride, 5 mM magnesium chloride, 2 mM EDTA and 5 mM sodium ascorbate, using a mortar and pestle with liquid nitrogen. As a phosphatase inhibitor, 10 mM sodium fluoride was added to the buffer. The homogenate was filtered through three layers of Miracloth (Calbiochem), and the pellets were washed three times with a buffer that comprised 50 mM HEPES (pH 7.6), 0.3 M sorbitol, 10 mM sodium chloride, and 5 mM magnesium chloride. Afterward, an aliquot of the thylakoid membrane extract containing 10 µg of chlorophyll was re-suspended in a buffer containing of 20% glycerol, 25 mMBis-Tris, and 10mM magnesium chloride (with 2% *n*-dodecyl-β-D-maltoside) for 10 min at 4℃ with gentle agitation. Each detergent-solubilized sample was mixed with a one-tenth volume of Serva Blue G buffer [100 mM Bis-Tris (pH 7.0), 0.5M aminocarporic acid, 30% (w/v) sucrose, and 50 mg ml^{-1} Serva Blue G (Serva)]. The samples were then centrifuged at 16,000xg for 20 min at 4℃. The solublized fractions were loaded on a 5%-to-13.5%-gradient BN-gel, and BN-PAGE was conducted at a constant current of 30 mA.

2.4. Histochemical assay for detection of superoxide

The accumulations of superoxide in the leaf segments was monitored by staining with nitroblue tetrazolium (NBT) as previously described (Fryer et al., 2002), but with some modifications. Briefly, the segments were infiltrated with

either a 6 mM NBT solution containing 50 mM HEPES buffer (pH 7.5) for 2 h in the dark. Afterward, they were illuminated at 1,500 µmol m^{-2}s^{-1} for 30 min. The segments were then immersed with 95% ethanol in a 65℃ water bath to extract all pigments. Finally, those accumulations were visualized based on segment staining and then photographed.

2.5. Histochemical assay for detection of hydrogen peroxide

The accumulations of hydrogen peroxide (H_2O_2) in leaf segments was monitored by staining with 3,3'-diaminobenzidine (DAB), respectively, as previously described (Fryer et al., 2002), but with some modifications. Briefly, the segments were infiltrated with 5 mM DAB solution containing 10 mM MES (pH 3.8) for 8 h in the dark. Afterward, they were illuminated at 1,500 µmol m^{-2}s^{-1} for 30 min. The segments were then immersed with 95% ethanol in a 65℃ water bath to extract all pigments. Finally, those accumulations were visualized based on segment staining and then photographed.

2.6. Analysis of ROS production by using antioxidant, SOD inhibitor in the leaf segments

To confirm the results for ROS production that were obtained by histochemical assays, it has investigated the effects of an antioxidant (5 mM ascorbic acid, or ASC) or an inhibitor of superoxide dismutase (SOD), i.e., 4 mM diethyldithiocarbamic acid (DDC), by adding the ASC or DDC to the histochemical assay solution for infiltration.

2.7. Quantification of singlet oxygen using fluorescence probes

Singlet oxygen production was detected by infiltrating the leaves with singlet oxygen sensor green (SOSG). Singlet oxygen production was monitored by using vacuum infiltration with 200 µM singlet oxygen sensor green (SOSG) (Dall'Osto et al., 2010). The SOSG fluorescence emission was measured at 530 nm with excitation at 480 nm before and after HL treatment at 1,000 µmol m^{-2}s^{-1} for 5 to 15 min.

2.8. Measurement of superoxide production in the thylakoid membrane of WT and *osstn8*

PSI- and PSII-driven superoxide production was monitored in thylakoid membrane using corresponding donor-acceptor pairs for PSI and PSII, respectively according to Zulfugarov et al. (2014). To detect levels of individual ROS generated by PSII-driven electron transport with a reaction buffer containing 0.1 M sucrose, 10 mM NaCl, 10 mM KCl, 5 mM MgCl$_2$, 10 mM Tricine, 1 mM KH$_2$PO$_4$ and 0.2% BSA, pH 8.0. The following ingredients were added to the reaction buffer immediately prior to the experiments: 30 mM sodium ascorbate to mediate deepoxidation of violaxanthin to zeaxanthin; 3 mM phenyl-p-benzoquinone to mediate PSII-driven electron transport and 0.3 mM ATP to fuel ATP hydrolysis. To detect levels of individual ROS generated by PSI-driven electron transport, again used the reaction mixture which contained 0.1 M sucrose, 10 mM NaCl, 10 mM KCl, 5 mM MgCl$_2$, 10 mM Tricine, 1 mM KH$_2$PO$_4$ and 0.2% BSA, pH (8.0). The following ingredients were added to the reaction buffer immediately prior to the experiments: 5 mM NH$_4$Cl as an uncoupler of oxygen-evolving complex, 20 µM 3-(4,4-dichlorophenyl)-1,1-dimethylurea (DCMU) as a

PSII electron transport inhibitor, 30 mM sodium ascorbate and 0.1 mM 2,6-dichlorophenol-indophenol as electron donor for PSI; 50 µM methyl viologen or 1 mM $NADP^+$ supplemented with 10 µM ferredoxin to mediate linear electron transport, and 0.3 mM ATP to fuel ATP hydrolysis. ROS sensors ie; 15 µM NBT was used to detect superoxide. In all experiments chlorophyll concentration was 10 µg per ml. Samples were illuminated at 700 µmol $m^{-2}s^{-1}$ for photoinhibition.

2.9. Sucrose density gradient ultracentrifugation

Sucrose density gradient ultracentrifugation (SDGU) was performed to isolate different CP complexes, including PSI and PSII complexes, as described previously (Dunahay and Staehelin, 1985). Aliquots of the thylakoid membrane extract containing 500 µg of chlorophyll were suspended in octylglucoside/sodium dodecyl sulfate (SDS). The solubilized fractions were then centrifuged at 100,000xg for 15 h at 4℃, using a SW41 Ti rotor (Beckman). Similarly, the LHCII, PSI, and PSII-LHCII supercomplex were isolated by SDGU according to a standard method of (Tokutsu et al., 2012). Aliquots of thylakoid membrane extract containing 400 µg of chlorophyll were suspended in 1% n-dodecyl-α-D-maltopyranoside. Unsolubilized fractions was removed by centrifuge at 18,000xg for 10 min at 4℃,The solubilized fraction was centrifuged at 90,000xg for 24 h at 4℃, on the SW41 Ti rotor.

Samples from these SDGU-isolated complexes were used for further experiments. The composition of each complex was verified by BN-PAGE and immunoblotting with appropriate antibodies against D1, Lhcb1, PsbS, PsaA/B proteins. The CP complex band, separated by SDGU, was mixed with a one-tenth volume of Serva Blue G buffer for BN-PAGE.

2.10. Measurement of active PSI complex of Sucrose density gradient ultracentrifugation

PSI activity of isolated complex of PSI of sucrose density gradient ultracentrifugation was measured by the method of (Bassi and Simpson, 1987) with some modification. About 2 µg of isolated complex of PSI of SDGU was used to check the active P700. About 2µg isolated PSI complex was added in reaction buffer (50 mM Tris-HCl, pH 8.8 supplemented with 0.05% Triton X-100). Now, 25 mM potassium ferricyanide (5 µl of a 5 mM solution per ml) was added to oxidize the sample then measured the absorbance at 700 nm. Similarly, 0.5 mM ascorbic acid (5 µl of 0.1 M ascorbic acid per ml) was added to reduce the sample then measured the absorbance at 700 nm. Hence the difference value of oxidation and reduction of the sample at 700 nm reveal that the isolated PSI complex was active.

2.11. Measurement of room temperature absorption spectra of isolated complexes of SDGU

Absorption spectra of isolated complex of sucrose density gradient ultracentrifugation were recorded at room temperature. Each complex of isolated samples (LHCII, PSI, PSII and PSII-LHCII supercomplex) was collected then absorption spectra were measured from 350 nm to 700 nm at room temperature by using Nano Drop ND-1000 spectrophotometer.

2.12. Measurement of chlorophyll *a/b* ratio

About 2 µg of isolated PSI and PSII complex was solublized in 2 ml 80%

acetone. The concentration of chlorophyll *a* and chlorophyll *b* was measured following the method of Porra et al. (1989). Chlorophyll *a/b* ratio was calculated by dividing the value of chlorophyll *a* by chlorophyll *b*.

2.13. Immunoblot analysis of thylakoid membranes and chloroplast protein complexes separated by SDGU

Immunoblotting assay in thylakoid membrane proteins was conducted as previously described (Towbin et al., 1979) that is described in the part I of this dissertation. Similarly Lhcb1, PsaA/B, and PsbS proteins were detected by using respective antibodies provided by Agrisera.

2.14. Confirmation of isolated complexes by SDGU with immunoblotting, BN-PAGE and coomassie staining

Representative complexes (LHCII, PSI and PSII-LHCII supercomplex) from SDGU for respective complex were loaded and separated by SDS gel. Immunoblotting was carried out according to previous method. D1 antibody was used to detect PSII core protein, Lhcb1, D1 and PsbS antibody was used to detect the marker protein of PSII-LHCII supercomplex, Lhcb1 antibody was used to detect a marker protein of LHCII and PsaA/B antibody was used to detect the marker protein of PSI. Similarly each complex was further confirmed by using 5-12.5% BN-PAGE. Isolated thylakoids were used as positive control. In addition the composition of polypeptides of LHCII, PSI and PSII-LHCII supercomplex were separated by SDS-PAGE and visualized by commassie blue staining.

2.15. Invitro protein phosphorylation in the thylakoid membranes

Leaf segments were taken and incubated at dark for 6 h. Thylakoid membranes were isolated in dim light condition according to Rintamäki et al., 1996 with following modifications. Leaf segments were ground in morter and pistle at $4^{o}C$ in isolation buffer [50 mM HEPES-NaOH, pH 7.5, 300 mM sucrose, 5 mM $MgCl_2$, 1 mM Na-EDTA, 10 mM NaF, and 1% (w/v) bovine serum albumin] then the homogenates was filtered through Miracloth and centrifuged at 3000 g for 5 min. The pellets were washed with washing buffer [10 mM HEPES-NaOH, pH 7.5, 5 mM sucrose, 5 mM $MgCl_2$, and 10 mM NaF] and centrifuged at 4000xg for 5 min. Thylakoid pellets were resuspended in a small amount of storage buffer [10 mM HEPES-NaOH, pH 7.5, 100 mM sucrose, 5 mM NaCl and 10 mM $MgCl_2$]. Chlorophyll concentration in thylakoid membrane was measured according to Porra et al. (1989). Invitro phosphorylation was performed according to Rintamäki et al. (2000). Thylakoid membrane aliquot that contains 10 µg chlorophyll was resuspended in assay buffer [50 mM Hepes-NaOH (pH 7.5), 100 mM sucrose, 5 mM NaCl, 10 MgCl2, and 10 mM NaF at a final chlorophyll concentration of 0.4 mg ml^{-1}. Finally 0.4mM ATP was added then thylakoid membrane aliquot was illuminated at 100 µmol $m^{-2}s^{-1}$ from 5 to 20 min at room temperature. After the illumination, the thylakoid sample was immediately centrifuged at 13000xg for 3 min then proteins were extracted by adding 1XSDS sample. Electrophoreses and immunoblotting was carried out that is explained in part in previous section or in part I. Finally invitro phosphorylation was carried out by using phosphothreonine antibody that can detect CP43, D1 and LHCII protein phosphorylation.

2.16. Measurements of superoxide production in isolated PSI, PSII, and PSII-LHCII supercomplex

Superoxide production in isolated CP complexes, i.e., PSI, PSII, and PSII-LHCII supercomplex, was determined with corresponding electron donors and acceptors as previously described (Zulfugarov et al., 2011; Zulfugarov et al., 2014). An aliquot of each complex containing 5 µg of chlorophyll and areaction buffer comprising 0.1 M sucrose, 10 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 10 mM Tricine, 1 mM KH_2PO_4, and 0.2% BSA (pH 8.0). The following additions were made immediately before the measurements were taken: 30 mM sodium ascorbate to mediate the de-epoxidation of violaxanthin to zeaxanthin; 3 mM phenyl-p-benzoquinone to mediate PSII-driven electron transport; or 0.3 mM ATP to fuel ATP hydrolysis. Superoxide production through PSI-driven electron transport was also measured in isolated CP complexes by using the same reaction buffer described above. For this, the following additions were made immediately before measurements were taken: 5 mM NH_4Cl as an uncoupler of the oxygen-evolving complex; 20 µM 3-(4,4-dichlorophenyl)-1,1-dimethylurea as a PSII electron transport inhibitor; 30 mM sodium ascorbate and 0.1 mM 2,6-dichlorophenol-indophenol as an electron donor for PSI; or 50 µM potassium ferricyanide supplemented with 0.3 mM ATP to fuel ATP hydrolysis. After 15 µM NBT was added, the samples were illuminated at 700 µmol $m^{-2}s^{-1}$ to promote photoinhibition. Hence, superoxide production in isolated CP complexes was evaluated by monitoring the changes in NBT absorbance at 560 nm (Auclair and Voisin, 1985), using a UV-1650PC UV-visible spectrophotometer (Shimadzu).

2.17. Analysis of protein oxidation in isolated complexes LHCII, PSII, PSII-LHCII supercomplex and PSI

Different chloroplast protein complexes ie; LHCII, PSII, PSII-LHCII supercomplex and PSI complex was used to detect the oxidation of respective protein complex. An equal amount of chlorophyll was taken and the overall oxidation of proteins was detected with an OxyBlot[TM] Protein Oxidation Detection Kit (Millipore), according to the manufacturer's instructions.

2.18. Analysis of photodamaged PSII-LHCII supercomplex of SDGU

Isolated PSII-LHCII supercomplex of SDGU sample of dark adapted and high light illuminated leaf segments of WT and *osstn8* were collected as mentioned above. Aliquot of PSII-LHCII supercomplex that contain 1µg chlorophyll were denatured with SDS sample buffer and polypeptides were separated by SDS-PAGE using 12% (w/v) acrylamide gel. Proteins were electrotransferred into nitrocellulose membrane (Amersham Hybond[TM]-P). Photodamaged of PSII-LHCII supercomplex was identified by detecting the damaged D1 protein on the basis of its degradation fragments using anti-D1 antibody as described previously by Miyao, (1994).

3. RESULTS

3.1. HL induced generation of superoxide and hydrogen peroxide from *osstn8* mutants and WT rice plants

As *osstn8* mutant leaves of rice are more susceptible to photoinhibition and assumed to accumulate more ROS when compared with WT leaves. The type and location of ROS generation in the mutants has been characterized. Histochemical staining with NBT and DAB revealed that more superoxide and H_2O_2 were produced in the mutant than in WT leaves under HL treatment (Fig. 20A, B). Although artifacts can cause problems during the time of histochemical assays, e.g., NBT acting as an electron acceptor for PSII (Zulfugarov et al., 2014; Halliwell and Gutteridge, 1989), so the effect of the antioxidant ASC and DDC, an inhibitor of the antioxidizing enzyme SOD is tested. When leaf segments were infiltrated with NBT or DAB plus ASC, less superoxide or $H_2O_2$was produced than in segments treated with NBT or DAB alone. This supported the conclusion from histochemical assays that less superoxide or H_2O_2 is accumulated in HL-treated tissues. Similarly, when DDC was added to the infiltration solution with NBT or DAB, superoxide production was enhanced (Fig. 20A, B) while H_2O_2 production declined (Fig. 20B) when compared with the controls. These results suggested that the increase in NBT staining was due to superoxide production that could be dismutated by SOD. Furthermore, the results from DDC-infiltration indicated that significantly more superoxide was initially produced in the mutant than in the WT plants, leading us to predict that the next event would probably be a SOD-related increase in H_2O_2. Previously it was reported that superoxide is produced earlier than H_2O_2 production which was delayed by 2~3 min (Nath et al., 2013b). Therefore, it can be proposed that superoxide is the more common form

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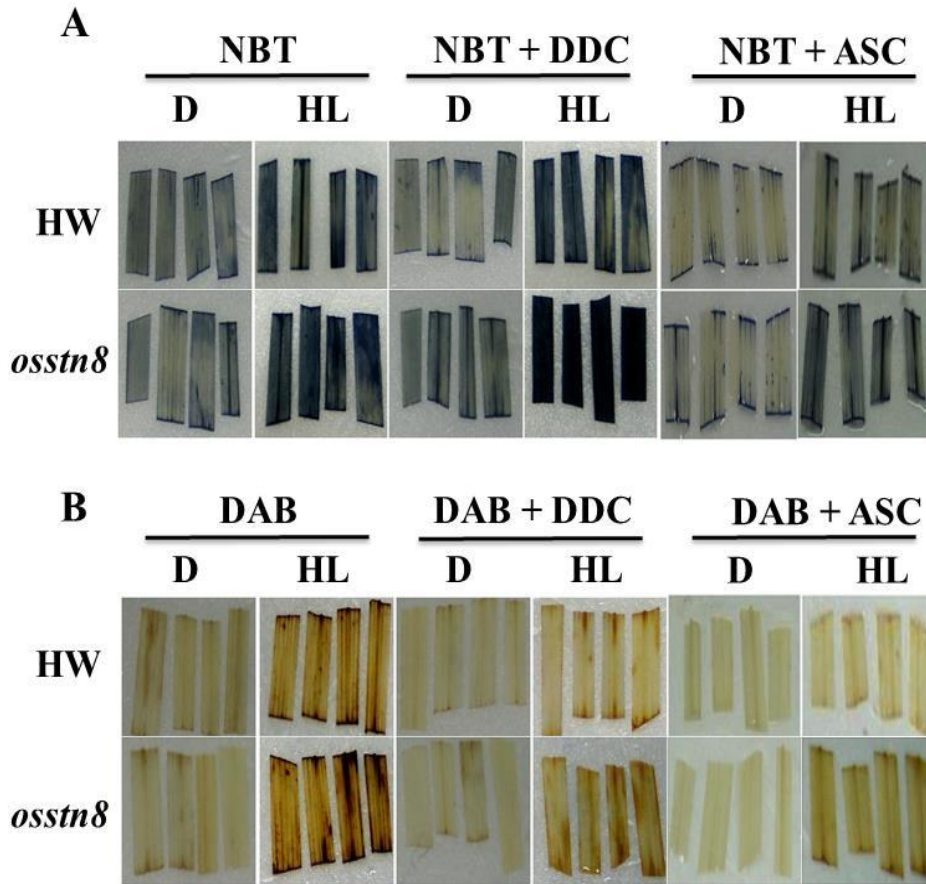
Figure 20. Measurements of ROS production in leaf segments from WT and *osstn8* plants. (**A**) Histochemical assay of superoxide using NBT in segments under high light (HL) treatment. To confirm that NBT staining was due to superoxide production, 5 mM ASC (as an oxidant) or 4 mM DDC (as a SOD inhibitor) was infiltrated along with NBT, (**B**) Histochemical assay of hydrogen peroxide using DAB in leaf segments under HL conditions. To test effect of ASC or DDC treatment, each chemical was infiltrated together with DAB.

of ROS produced in the mutants.

3.2. More superoxide was produced in the thylakoid membrane of *osstn8* rice mutants than in the WT plants under HL conditions

Thylakoid membranes were isolated from dark adapted leaf sample and superoxide production was measured by using NBT. The result reveals that superoxide production in PSII driven superoxide production was higher in *osstn8* mutant (Fig. 21A), although superoxide production in total ETR was similar between WT and *osstn8* mutant (Fig. 21B). However, superoxide production in PSI driven ETR was significantly less in both WT and *osstn8* mutant (Fig. 21C). This result suggests that superoxide production was higher in *osstn8* mutant where PSII core protein phosphorylation was block. Similarly, phosphorylation of PSII core proteins was analyzed to confirm whether they can phosphorylate during the time of measurement of superoxide. Hence the in vitro phosphorylation assay has done and interestingly it has observed that PSII core protein phosphorylation was observed when thylakoid membranes were illuminated with the addition of ATP (Fig. 21D).

3.3. Singlet oxygen production was similar between WT and *osstn8* mutant

Singlet oxygen production was monitored in the leaf segments of WT and *osstn8* mutant. After imposing photoinhibitory illumination for 15 min, it is found this result to be interesting because it implied that singlet oxygen production in HL-treated leaves was not any higher in the *osstn8* mutant than in the WT plants, even though leaves of the former are more susceptible to strong irradiance
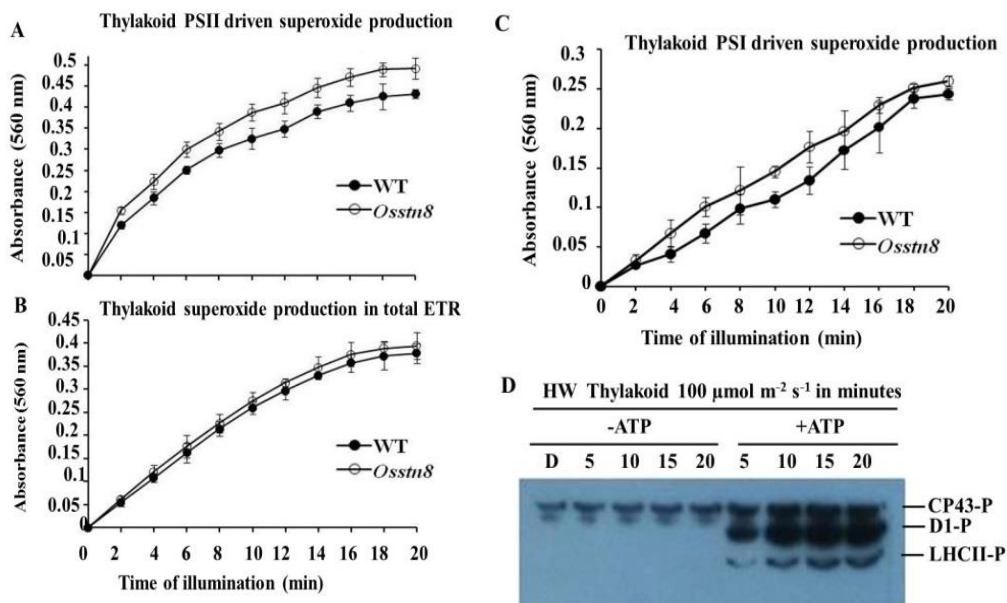
Figure 21. Measurements of superoxide production in isolated thylakoid membranes from WT and *osstn8* plants and subsequent *in vitro* phosphorylation of thylakoid membrane proteins. (**A**) PSII driven superoxide production in the thylakoid membrane of WT and *osstn8*, (**B**) Whole chain-driven superoxide production in the thylakoid membrane, (**C**) PSI driven superoxide production in the thylakoid membrane, (**D**) Analysis of *in vitro* protein phosphorylation in the thylakoid membrane of WT and *osstn8*. Thylakoid membranes were isolated in dim light condition from dark adopted leaf samples. In vitro protein phosphorylation was conducted by illumination with or without addition of ATP in the aliquot.
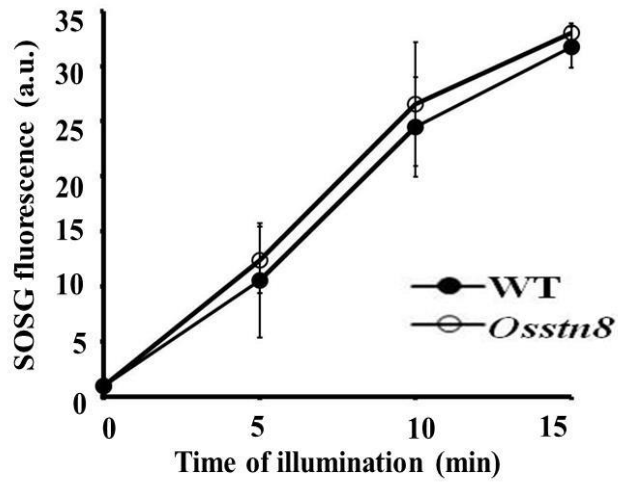
Figure 22. Measurement of singlet oxygen production in WT and *osstn8* leaf.
Time course of singlet oxygen generation in leaf segments was monitored as
increase in SOSG fluorescence emission at 530 nm with excitation at 480 nm at
room temperature. SOSG solution was infiltrated in the leaf segments of WT and
osstn8. For evaluating effect of HL treatment, leaf segments were illuminated at
1,000 µmol m^{-2}s^{-1} for 15 min and SOSG fluorescence emission was recorded.

(Nath et al., 2013b). To confirm this, the fluorescence emission of SOSG between the WT and the *osstn8* mutants after HL treatment was compared. Although those emissions were increased by illumination, singlet oxygen generation did not differ significantly between the WT and the *osstn8* mutants (Fig. 22). Depending upon the type of fluorescence probe, the average amount of singlet oxygen produced in the mutant was slightly less when treated with DanePy by Nath et al. (2013b) or slightly more when treated with SOSG (Fig. 22), although the differences were not significant.

3.4. More superoxide was produced from PSII in *osstn8* mutants than in the WT plants

Because the oxidation of thylakoid proteins seemed to be more pronounced in PSII proteins (Part I, Fig. 19), hence PSI and PSII complexes were separated by SDGU (Fig. 23A). Before the measurement of superoxide production in isolated complex, the activity of PSI complex was measured by using potassium ferricyanide and ascorbic acid. Potassium ferricyanide oxidized PSI and the subsequent addition of ascorbic reduced PSI hence the difference in the absorption of PSI is known as active PSI (Fig. 23B), therefore isolated PSI complex was active during the time of superoxide production measurement. The composition of each complex was further confirmed by Coomassie staining of polypeptides separated by SDS-PAGE as well as by immunoblotting of the SDS-PAGE gels with antibodies raised against their specific proteins, chlorophyll *a/b* ratio, or by measuring absorption spectra (Fig. 24A, B, C, D, E, F).

Production of superoxide from isolated complexes under HL conditions (illumination at 700 µmol m^{-2}s^{-1}) was recorded as the increase in absorbance of
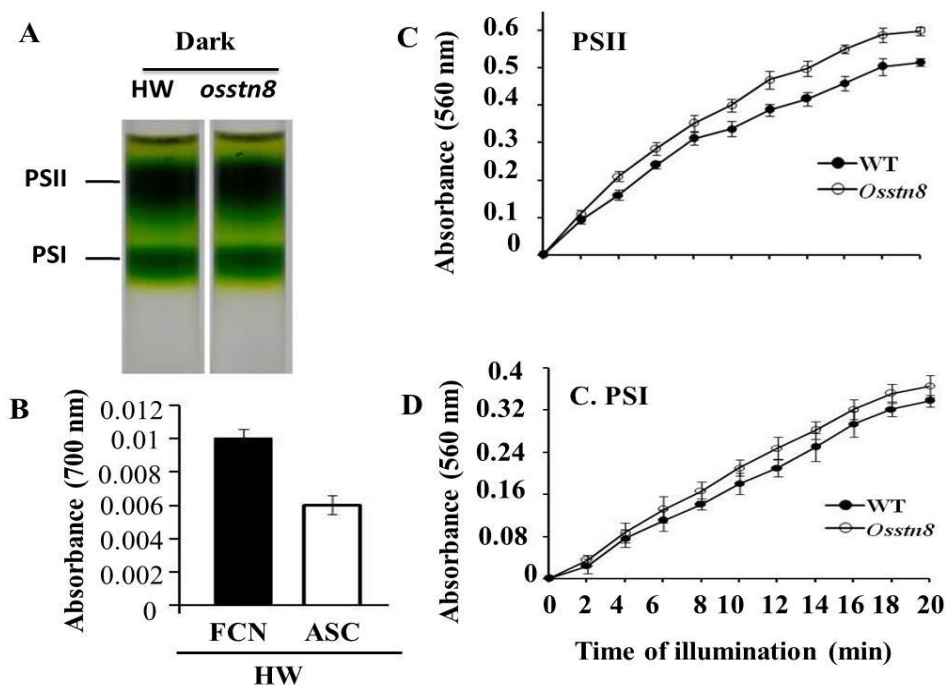
Figure 23. Separation of PSI and PSII complexes by sucrose gradient ultracentrifugation in WT and *osstn8* and subsequent measurements of reactive oxygen species (ROS). (**A**) Isolation of PSI and PSII complex in WT and *osstn8* mutant. Thylakoid membranes were isolated from dark adapted leaves sample of WT and *osstn8* mutant and subjected to sucrose density gradient ultracentrifugation. (**B**) Measurement of PSI activity in isolated PSI complex of sucrose gradient WT sample, (**C**) Aliquot of PSII complex from WT and *osstn8* was taken and superoxide production was measured by PSII driven superoxide production, (**D**) Aliquot of PSI complex from WT and *osstn8* was taken and superoxide production was measured by PSI driven superoxide production. In Fig. B and C, aliquot of PSI and PSII complex that contain 10 µg chlorophyll dark samples of PSI and PSII and NBT absorbance changes at 560 nm for WT and *osstn8* was measured after HL at 700 µmol m^{-2}s^{-1} up to 20 min of illumination.

NBT at 560 nm. Here, the amount of superoxide from the isolated PSII complex was greater in the *osstn8* (Fig. 23C). However, the difference between the WT plants and the *osstn8* mutants was not significant from the isolated PSI complex when compared with values obtained for production from the PSII complex (Fig. 23D).

At the thylakoid level, the greater production of ROS in the mutants was confirmed by comparing the oxidation of thylakoid proteins between the WT and mutant leaves during 3 h of HL treatment at 1,500 µmol m^{-2}s^{-1}. In the *osstn8* plants, PSII core proteins seemed to be carbonylated more rapidly than in the WT plants (Part I, Fig. 19). The oxidation of thylakoid membrane proteins under HL conditions has also been shown in Arabidopsis *stn7xstn8* double mutants where PSII core protein phosphorylation was completely blocked (Tikkanen et al., 2008).

3.5. PSII proteins were more oxidized than PSI protein in *osstn8* mutants than in the WT

To test this further, WT and *osstn8* leaves were illuminated to HL then PSI and PSII complex were isolated by SDGU (Fig. 25A). Protein oxidation was compared between the WT and *osstn8* mutant plants with respect to the oxidation of thylakoid proteins in each PSI or PSII complex (Fig. 25B, C). These results clearly indicated that PSII proteins were preferentially oxidized to a greater extent when compared with PSI proteins, and that PSII proteins were more oxidized in *osstn8* leaves than in WT leaves.
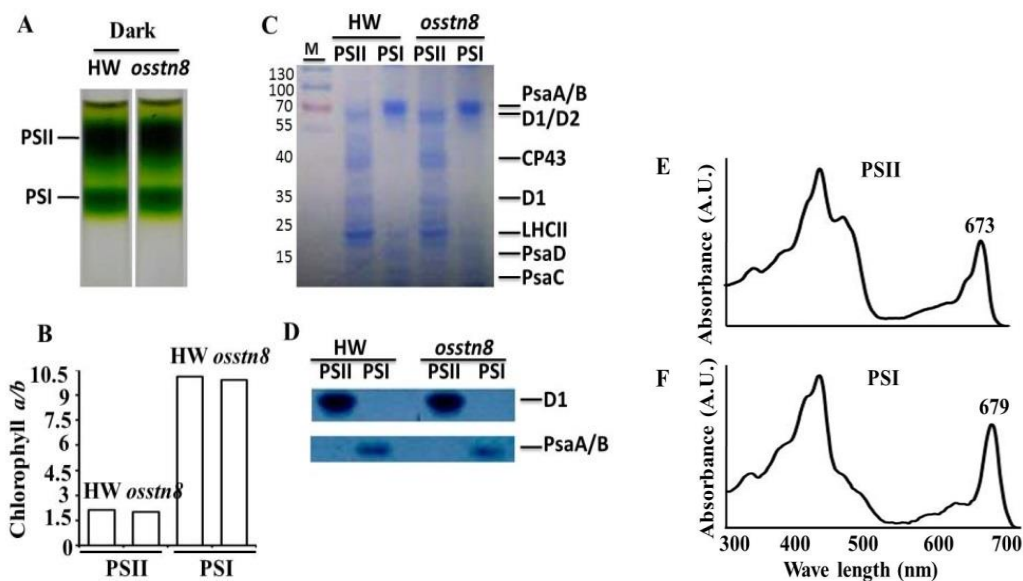
Figure 24. Confirmation of sucrose gradient samples of PSI and PSII. (**A**) Isolated PSI and PSII complex shown in Figure 23A, (**B**) Chlorophyll *a/b* ratio of PSI and PSII comples of WT and *osstn8*, (**C**) Protein profile of PSI and PSII complex of WT and *osstn8* sample of sucrose gradient where kDa represents the protein marker size in kilodalton, (**D**) Immunoblotting detection of major proteins of thePSI and PSIIcomplexes obtained by SDGU. D1 antibodywas used as marker proteins for PSIIcomplex and PsaA/B antibody was used as a marker protein for PSI complex. (**E, F**) Room temperature absorption spectrum of PSI and PSII complex that was isolated by SDGU was recorded. (**E**) Absorption spectra of PSII showing maximum absorbance at 673nm, (**F**) Absorption spectra of PSI showing maximum absorbance at 679 nm wavelength.

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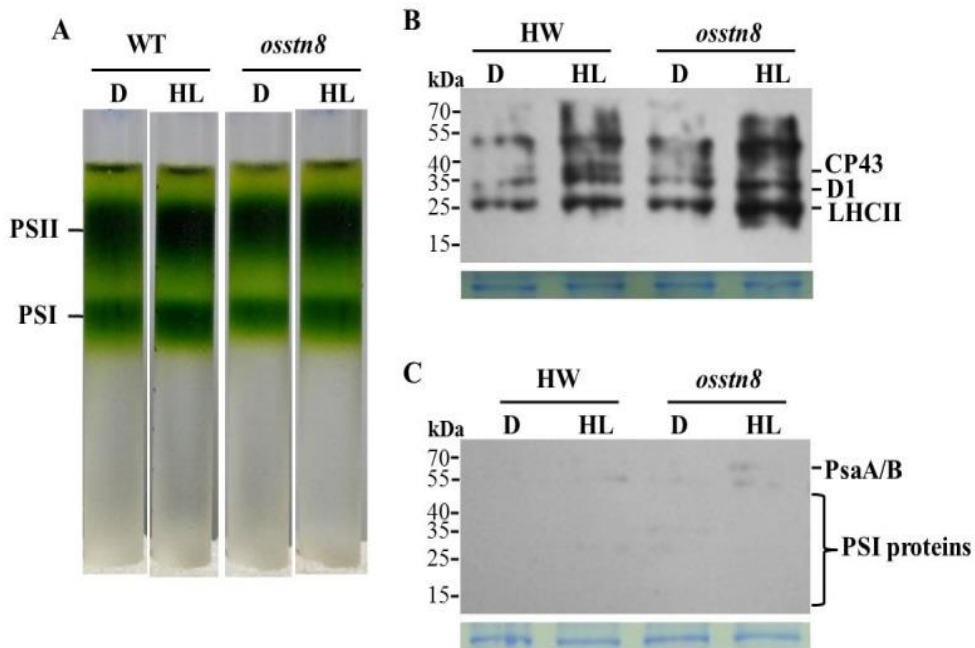
Figure 25. Separation of PSI and PSII complexes by sucrose gradient ultracentrifugation in WT and *osstn8* after high light illumination and subsequent detection of oxidation of proteins. (**A**)Thylakoid membranes isolated after dark and high light illumination from WT and *osstn8* mutant plants were subjected to sucrose density gradient ultracentrifugation. Isolated PSI and PSII complexes are shown. (**B**,**C**) Detection of oxidation of PSI and PSII proteins in WT and *osstn8.* PSI and PSII complexes of sucrose gradient sample containing 5 µg of chlorophyll were loaded in each lane. Oxidation status of proteins in PSII complex (**B**) and oxidation status of proteins in PSI complex (**C**), Protein marker is shown in the left side of the figure. The major LHCII proteins for PSII complex and PsaA/B protein for PSI complex in SDS-PAGE results after coomassie blue staining (CBS) is shown in the bottom panel for equal loading. D, dark-adaptedleaf segments for 12 h; HL, leaf segments illuminated at 1,500 µmol m^{-2}s^{-1} for 2 h, kDa, represents the protein size marker in kilodaltons.

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3.6. Preferential production of superoxide in PSII-LHCII supercomplex isolated from *osstn8* leaves

Because the mobilization of PSII-LHCII supercomplex was suppressed in HL-treated *osstn8* leaves (Part I, Fig. 14A, B), hence superoxide production in that complex has analyzed. As shown in Fig. 26A, LHCII, PSI, and PSII-LHCII supercomplex were separated by SDGU from WT and *osstn8* leaves and their individual identities were confirmed by BN-PAGE (Fig. 27A), by immunoblotting of the SDS-PAGE with antibodies raised against several proteins specific to each complex (Fig. 27B), by Coomassie staining of polypeptides separated by SDS-PAGE (Fig. 27C) and the absorption spectra were measured in each isolated complex (Fig. 27D, E, F).

Production of superoxide from isolated PSII-LHCII supercomplex under HL conditions (illumination at 700 µmol m^{-2}s^{-1}) was recorded as the increase in absorbance of NBT at 560 nm. Here, values were higher from *osstn8* mutant leaves than from the WT leaves (Fig. 26C). However, the difference in productivity between WT and *osstn8* plants was not significant from the isolated PSI-LHCI complex when compared with the PSII complex (Fig. 26D). The activity of isolated complex of PSI was measured by using potassium ferricyanide and ascorbic acid to oxidize and reduce PSI respectively. The difference in the absorbance in Fig. 26B showed that PSI complex was active during the time of measurement of superoxide production in Fig. 26C, D.

3.7. PSII-LHCII supercomplex proteins were more oxidized than PSI protein in *osstn8* mutants than in the WT

For further investigation, leaf samples placed under HL treatment then

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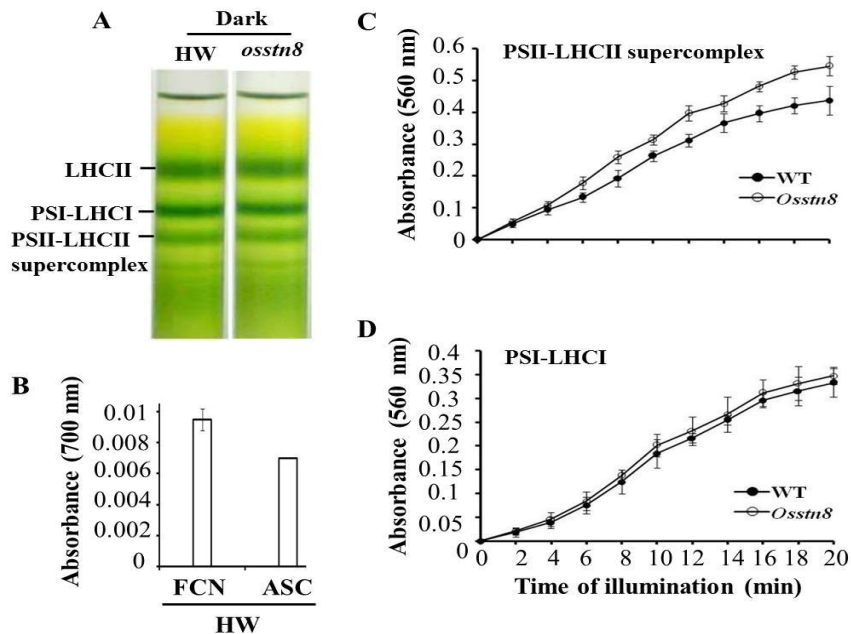
Figure 26. Separation of LHCII, PSI and PSII-LHCII supercomplex by sucrose gradient ultracentrifugation in WT and *osstn8* and subsequent measurement of reactive oxygen species (ROS). (**A**) Isolation of LHCII, PSI and PSII-LHCII supercomplex in WT and *osstn8* mutant. Thylakoid membranes were isolated from dark adopted leaves sample of WT and *osstn8* mutant and subjected to sucrose density gradient ultracentrifugation. (**B**) Measurement of PSI activity in isolated PSI complex of sucrose gradient WT sample, (**C**) Aliquot of PSII-LHCII supercomplex from WT and *osstn8* was taken and superoxide production was measured by PSII driven superoxide production, (**D**) Aliquot of PSI-LHCI complex from WT and *osstn8* was taken and superoxide production was measured by PSI driven superoxide production. In fig C and D, about aliquot of each complex that contain 10 µg chlorophyll was used from dark samples of PSI-LHCI and PSII-LHCII supercomplex and NBT absorbance changes at 560 nm for WT and *osstn8* was measured after HL at 700 µmol m^{-2}s^{-1} up to 20 min of illumination.
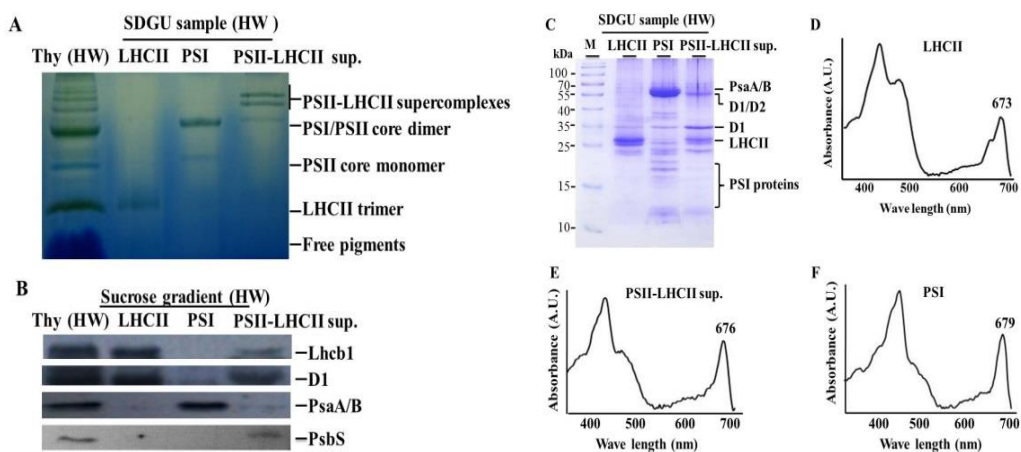
Figure 27. Confirmation of sucrose gradient samples of LHCII, PSI and PSII-LHCII supercomplex. Sucrose gradient samples LHCII, PSI and PSII-LHCII supercomplex from WT plants in Figure 26A was confirmed by using following methods. (**A**) BN-PAGE analysis of each complexeobtained by sucrose gradient from WT samples. Each complexes obtained by sucrose gradient were separated by 5-12.5% BN-PAGE. Isolated thylakoids from WT (Thy WT) were used as a positive control, (**B**) Immunoblotting detection of major proteins of the LHCII, PSI and PSII-LHCII supercomplex obtained by SDGU. Lhcb1, D1 and PsbS antibodies were used as marker proteins for LHCII and PSII-LHCII supercomplex and PsaA/B antibody was used as a marker protein for PSI complex, (**C**) Protein profile of LHCII, PSI and PSII-LHCII supercomplex of WT sample of sucrose gradient where kDa represents the protein marker size in kilodalton, (**D-F**) Room temperature absorption spectra of LHCII, PSI and PSII-LHCII supercomplex that was isolated by SDGU was recorded. (**D**) Absorption spectra of LHCII showing maximum absorbance at 673nm wavelength, (**E**) Absorption spectra of PSII-LHCII supercomplex showing maximum absorbance at 676 nm wavelength, (**F**) Absorption spectra of PSII-LHCII supercomplex showing maximum absorbance at 679 nm wavelengths.

then PSI, LHCII, PSII-LHCII supercomplex were isolated by SDGU (Fig. 28A). Each complex was taken and oxidation of proteins were compared between WT and *osstn8* mutant as before, proteins in PSII-LHCII supercomplex were preferentially oxidized to a greater extent in response to HL when compared with PSI proteins. Furthermore, PSII proteins, especially those in PSII-LHCII supercomplex, were preferentially more oxidized in *osstn8* leaves than in WT (Fig. 28B). The oxidation of PSI proteins was undetectable and the oxidation of LHCII proteins was similar between WT and *osstn8* during high light illumination (Fig. 28C, D).

Overall, these data demonstrated that the accumulation of damaged-but-unphosphorylated PSII in PSII-supercomplex may enhance the production of ROS, especially superoxide, in *osstn8* leaves under excess illumination. The level to which D1 fragments accumulated was less in PSII-LHCII supercomplex of the *osstn8* mutants when compared with that supercomplex in WT plants (Fig. 28E). This indicated that the damaged and phosphorylated PSIIs in the supercomplex had already started to become fragmented by proteases even while being mobilized in WT plants. In contrast, this process was completely blocked in the *osstn8* mutants.
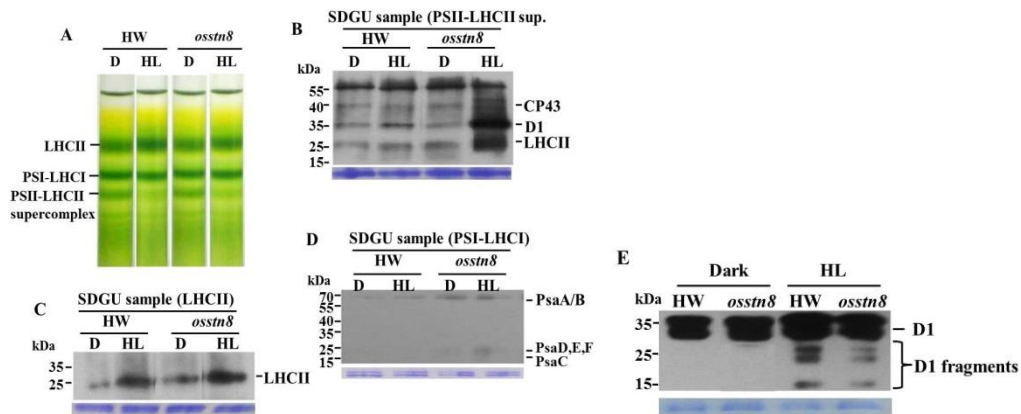
Figure 28. Separation of LHCII, PSI and PSII-LHCII supercomplex by sucrose gradient ultracentrifugation in WT and *osstn8* after high light illumination and subsequent detection of oxidation of proteins. (**A**) Thylakoid membranes were subjected to sucrose density gradient ultracentrifugation to isolate LHCII, PSI and PSII-LHCII supercomplex, (**B**) Oxidation status of proteins in PSII-LHCII supercomplex. Coomassie staining of membrane showing LHCII is shown in the bottom for loading control, (**C**) Oxidation status of proteins in LHCII complex, Coomassie staining of membrane showing LHCII is shown in the bottom for loading control, (**D**) Oxidation status of proteins in PSI complex, Coomassie staining of membrane showing PsaA/B protein is shown in the bottom for loading control. In (**B**-**D**), aliquot of each complex that containing 5 µg of chlorophyll were loaded in each lane and (**E**) Detection of D1 protein damaged in PSII-LHCII supercomplex from WT and *osstn8* plants. An aliquot of PSII-LHCII supercomplex containing 1µg chlorophyll was loaded in each lane. Immunoblot analysis of D1 protein and its degradation fragments was performed using anti-D1 antibody. Major LHCII proteins separated by SDS-PAGE after Coomassie blue staining (CBS) shown in bottom panel were used for equal loading. D, leaf segments dark-adapted for 6 h; HL, segments illuminated at 1,500 µmol m^{-2}s^{-1} for 2 h.

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4. DISCUSSION

Several approaches were performed to determine the major site for the production of ROS, especially superoxide, in *OsSTN8* knock-out rice leaves under HL illumination. The results showed that the site of ROS production is PSII. As shown by spectrophotometric assay of NBT absorbance, PSII particles produced more superoxide in *osstn8* mutants than in the WT plants. When PSII-LHCII supercomplex was separated by SDGU, it produced more superoxide in the mutants than in the WT plants, but differences between PSI particles from the two types of plants were not significant. The results are in contrast to PSII-related superoxide production between mutant and WT plants, which had been examined by protein oxidation assays of PSII particles in *osstn8* mutants under HL treatment. In addition, the protein oxidation assays showed that PSII-LHCII supercomplex proteins were more oxidized in the mutant (Fig. 28B), implying that PSII particles in that supercomplex were more damaged even though their degradation step was partially blocked. All of these findings provided evidence that the damaged PSII in PSII-LHCII supercomplex is the major source of superoxide in *osstn8* mutants upon exposure to excess illumination.

Among the ROS, singlet oxygen either can be quenched by β-carotene or α-tocopherol or otherwise can react with the D1 protein as a target (Krieger-Liszkay, 2004; Noguchi, 2002; Nishiyama et al., 2001). In the present stusy, singlet oxygen did not accumulate to a greater extent in *osstn8* mutants than in the WT plants under HL treatment. Under photoinhibitory illumination, H_2O_2 was reported to damage cells and cause oxidative bursts, leading to cell death and interfere with the transcription of D1 protein in the PSII repair cycle (Nishiyama et al., 2004; Nishiyama et al., 2006). In this study, *osstn8* mutant was found to

produce H_2O_2 and superoxide, probably due to failure in the phosphorylation of damaged PSII core proteins. Previously it has learned that, in *osstn8* mutant of rice, this accumulation begins earlier for superoxide than for H_2O_2 (Nath et al., 2013b). Together with this, the results with a SOD inhibitor DDC indicate that, in the mutants, superoxide is the major form of ROS produced.

Not only are ROS toxic but they can also function as signaling molecules (Mittler et al., 2004; D'Autréaux and Toledano, 2007; Foyer and Shigeoka, 2011; Schmitt et al., 2014). They are produced at various locations within the cells, including mitochondria, peroxisomes, glyoxysomes, plasma membranes, endoplasmic reticulum, cell walls, and chloroplasts (Gechev et al., 2006; Das and Roychoudhury, 2014). Many enzymatic sources of ROS production have been found in those organelles: cell wall-bound peroxidases, including apoplastica peroxidases (Minibayevaet al., 1998; Minibayeva et al 2009; Bolwell et al., 2002); plasma membrane NADPH oxidases (Sagi and Fluhr, 2001; Mori and Schroeder, 2004; Sagi and Fluhr, 2006) and oxalate oxidase (Svedružić et al., 2005); apoplastic polyamine oxidases and oxalate oxidase; peroxisomal flavin oxidases (Das and Roychoudhury, 2014); and amino oxidases (Angermüller et al., 2009). In the chloroplasts, PSI is considered the main site of superoxide generation in the thylakoids under photoinhibitory illumination (Ogawa et al., 1995; Asada, 1999; Gechev et al., 2006; Sonoike 2006, Miyake, 2010). Therefore, if most of the superoxide is generated from PSI, it can be expect that, under HL conditions, PSI should be more severely damaged in *osstn8* mutants than in WT plants. However, in this study PSI activity was found to be similar between both genotypes (Part I, Table 2). Light-induced generation of superoxides has been found on both sides of electron acceptor and electron donor of PSII (Ananyev et al., 1994; Cleland and Grace, 1999; Pospíšil et al., 2006; Pospíšil, 2009; Pospíšil, 2012). Cytochrome b_{559} may also be involved in this generation, as proposed by the

reports that superoxide radicals can be located by electron paramagnetic resonance (EPR) spectroscopic analysis of PSII particles isolated from a cytochrome b_{559} mutant of tobacco (Pospíšil et al., 2006). The detection of superoxide in isolated thylakoids by a voltammetric method suggests that, when the photosynthetic electron transport chain is over-reduced, superoxide may be generated at the site of Q_A, the primary electron acceptor of PSII (Cleland and Grace, 1999). Because phosphorylation affects the conformation and organization of PSII supercomplex (Kiss et al., 2008; Kereïche et al., 2010; Lambrev et al., 2010; Zulfugarov et al., 2010).

In comparisons of *osstn8* and WT leaves, it is calculated more superoxide production in PSII for the mutant, but the same was not true in PSI. However, it is not compare the amounts of superoxide produced by PSII and PSI. Researchers have also questioned the role of the Mehler reaction as a major sink for excess excitation energy when CO_2 assimilation is restricted in C_3 and Crassulacean acid metabolism plants (Badger et al., 2000; Driever and Baker, 2011). Furthermore, it is not observe that PSI did not seem to incur more photodamage in treated plants, and also observed superoxide production in PSII particles, especially in PSII-LHCII supercomplex. Recently it is reported that, when compared with the WT plants, more superoxide is produced in PSII of PsbS knock-out rice plants lacking a major component of non-photochemical quenching, qE (Johansson et al., 2013; Zulfugarov et al., 2014). However, the major site of superoxide production in those knock-out mutants might be different from that of *osstn8* plants. Both the conformation and organization of PSII supercomplex affect superoxide production in PSII (Zulfugarov et al., 2014). Phosphorylation of PSII proteins increases membrane fluidity and, hence, the mobility of the protein complex within the thylakoid membrane when plants are exposed to strong irradiance (Goral et al., 2010; Herbstova et al., 2012). For STN mutants, it is expected their

membrane fluidity and mobility of CP complexes to decrease, thereby leading to ROS production. Therefore, the lack of any conformational changes due to STN8 kinase-induced phosphorylation in *osstn8* mutants may have been related to the reason for more superoxide production in those particular plants.

Finally, protein oxidation assay of CP complexes isolated by SDGU clearly demonstrated that superoxide production from PSII particles is greater in *osstn8* mutants than in the WT plants. Furthermore, PSII-LHCII supercomplex cannot be mobilized to the grana margin for degradation of damaged proteins, and the damaged PSII in PSII-LHCII supercomplex of *osstn8* mutants is the major source of superoxide.

Part III

Role of CP29 and LHCII Phosphorylation during State Transition and Supercomplex Mobilization

ABSTRACT

Unlike with Arabidopsis, spinach and cucumber, CP29 protein phosphorylation (CP29-P) was easily observed in rice when exposed to high light (HL) or light chilling treatment by using immunoblotting assay. The result indicates that monocots and dicot plants seems to have a difference in the capacity to phosphorylate CP29. To clarify corresponding kinase and phosphatase for reversible CP29-P are still unknown, T-DNA insertion knock-out mutants of rice STN7 and STN8 kinase as well as PPH phosphatase mutants were used to study the reversible phosphorylation of CP29. Upon exposure to high light or light chilling treatment, CP29-P was observed in WT, *osstn7* and *ospph* mutant but not in *osstn8* mutant. In addition, CP29-P was also observed in *osstn8* complement plant. However dephosphorylation of CP29-P was observed in all genotypes during dark incubation. The phosphorylation of LHCII protein was blocked in *osstn7* mutant and dephosphorylation of LHCII was blocked in *ospph* mutant, as a consequence, state transition was strongly impaired. Mobilization of PSII supercomplex was observed in *osstn7* mutant during high light illumination but it was suppressed in *ospph* mutant. This result proposed that dephosphorylation of LHCII protein was also essential for mobilization of PSII

supercomplex. The relative change in maximal fluorescence during state transition, ΔF was similar in WT and *osstn8* mutant during low blue light and far red light illumination. And, mobilization of PSII supercomplex was not observed in both genotypes. However, ΔF became negligible in *osstn8* mutant but not in WT during high light illumination and mobilization of PSII supercomplex was only observed in WT. Upon high light illumination, CP29-P was observed in PSI complex in WT but not in *osstn8* mutant. Finally, it can be proposed that lack of CP29-P also impaired high light induced state transition in *osstn8* mutant of rice.

1. INTRODUCTION

Photosystem II (PSII) is surrounded by LHCII proteins with outer trimeric and inner monomeric antenna proteins (Dekker and Boekema, 2005). Peripheral antenna proteins bind with both side of PSII core and each consists of one LHCII trimer and two LHCII monomers in higher plants (Boekema et al., 1995). Additionally, CP29 encoded by Lhcb4 and CP26 encoded by Lhcb5, are minor monomeric LHCII proteins among higher plants and green algae while another minor monomeric LHCII protein, CP24 encoded by Lhcb6 is present only in higher plants (Minagawa and Takahashi, 2004). CP29, a light harvesting complex in photosystem II of plants, plays multiple putative roles in vivo: in addition to collecting solar energy, its location at the periphery of the reaction center core implies that the excitation originating in other regions of the photosynthetic antenna must be channeled through it. Furthermore, it has been implicated in the non photochemical quenching of excess chlorophyll excitation in high light conditions for the protection of plants from oxidative stress (Ahn et al., 2008).

There are several PSII proteins such as D1, D2, CP43, CP47, PsbH, CaS, TSP9, light harvesting complex (Lhcb1, Lhcb2) and CP29 that are phosphorylated under different environmental conditions (Bennet, 1980; Carlberg et al., 2003; Fristedt et al., 2010; Chen et al., 2009; Bellafiore et al., 2005; Samol et al., 2012). Whereas STN8 kinase phosphorylates PSII core protein and STN7 kinase phosphorylates LHCII protein in Arabidopsis (Bonardi et al., 2005). Some result suggest that, minor light-harvesting antenna protein CP29 phosphorylated in C3 plant (barley) and C4 plant (maize) but not in spinach during water stress (Liu et al., 2009) or salt and cold stress (Pursiheimo et al., 2003). The phosphorylation of CP29 can modulate the spectral properties of photosystem II

subunit and provide a possible mechanism for the regulation of excitation energy supply to the reaction center (Croce et al., 1996).

Tikkanen et al. (2006) observed the phosphorylation of CP29 protein isoform Lhcb4.2 by STN7 kinase during PSII light illumination in Arabidopsis. There are other reports claiming that phosphorylation of CP29 are common in monocots but not in dicots (Chen et al., 2009), and that CP29 phosphorylation plays a role in state transition in *Chlamydomonas* (Tokutsu et al., 2009). Later, Frietedt and Vener (2011) observed high light induced disassembly of PSII supercomplex required phosphorylation of CP29 by STN7 kinase in Arabidopsis. Recently, it has been demonstrated that the phosphorylation of CP29 protein is independent of STN7 kinase in rice (Betterle et al., 2015). In order to address those controversies, phosphorylation of CP29 was investigated between dicot and monocot plants. Finally, it has identified the kinase that regulates the phosphorylation of CP29 during high light and light chilling treatment in rice. The present study successfully demonstrated that STN8 kinase was responsible for phosphorylation of CP29 protein in rice plants during high light and light chilling treatment. These results also suggested that the two stages of state transition ie; state transition 1 [ST1] and state transition 2 [ST2] due to the phosphorylation of LHCII and CP29 antennae proteins phosphorylation.

2. MATERIALS AND METHODS

2.1. Plant materials and growth conditions

From a T-DNA inserted mutant pool, *OsSTN8* knock-out mutant *osstn8* was used as described in Nath et al., 2013b. Wild-type (WT) rice (*Oryza sativa* cultivar 'Hwayoung-byeo', HW), *osstn8* mutant and *osstn8* complemented line 1 (Comp-1) were used according to Nath et al. (2013b). *OsPSBS* knock-out mutant *osPsbS* were used according to Zulfugarov et al. (2014). Similarly, Wild-type rice (*O. sativa* L. *japonica* cultivar 'Nipponbare', NIPP), and *OsSTN7* knock-out mutant *osstn7*, were kindly provided by professor Roberto Bassi, University of Verona, Italy (Betterle et al., 2015) were screened from a T-DNA inserted mutant pool from Oryza Tag line at http://oryzatagline.cirad.fr/. The gene name of *osstn7* is AJTH05. For *OsPPH* mutant, Wild-type rice (*O. sativa* L. *japonica* cultivar 'Zhonghua 11', ZH11), and *OsPPH* knock-out mutant *ospph*, also kindly provided by professor Roberto Bassi, University of Verona, Italy (Betterle et al., 2015) were screened from T-DNA inserted mutant pool from Rice Mutant Database at http:// http://rmd.ncpgr.cn/. The gene name of *ospph* is 04Z11OK94. All plants were grown in a greenhouse at $30/26^{o}C$ in the 16/8 h day/night photoperiod.

2.2. Photoinhibitory and light chilling treatment

Leaves were chosen from WT, *osstn8*, *osstn7* and *ospph* mutant plants. About 2-3 cm leaf segments were prepared in water to protect against severe wounding, and then floated on water. The segments were incubated at dark for overnight before high light (HL) and light chilling (LC) treatment. For photoinhibitory treatment, leaf segments were placed under 1,500 µmol $m^{-2}s^{-1}$

provided with a metal halide lamp equipped with a water bath to reduce heat. Leaf segments were illuminated at 200 µmol m^{-2}s^{-1} for 2 h for low light illumination. Similarly light-chilling treatment was applied at 4°C in the low light for several time periods that is mentioned in the figure legends.

2.3. Measurement of photochemical efficiency of PSII

Room temperature chlorophyll fluorescence parameters including minimum fluorescence (Fo) and maximal fluorescence (Fm) from detached leaf segments were measured using PAM 2000 (Walz Effeltrich, Germany) following the manufacturer's instructions after dark incubation for 30 min. The photochemical efficiency of PSII, or Fv/Fm, was calculated using the equation, Fv/Fm = (Fm - Fo) / Fm.

2.4. Isolation of thylakoid membrane and immunoblotting

Thylakoid membranes were isolated following the protocol described by (Oh et al., 2009), and 10 mM NaF was used during the time of isolation of thylakoid membranes. Chlorophyll concentration in isolated thylakoids was measured following the method of Porra et al., 1989. The procedure for isolation of thylakoid membrane and immunoblotting has been described in the part I of this dissertation. Finally, D1 antibody, phosphothreonine antibody, Lhcb1 antibody, PsaA/B antibody, PsbS antibodies etc., were used for immunoblotting assay for respective proteins. The phosphorylation of CP29 protein was detected by using CP29 antibody (Hwang et al., 2003) and phosphorylation of PSII and LHCII proteins was detected using phosphothreonine antibody (Cell Signalling).

2.5. Blue native gel electrophoreses (BN-PAGE)

Two different methods of BN-PAGE were performed to isolate different chloroplast protein (CP) complexes. Method 1, separation of different CP complex with PSII-LHCII supercomplex. BN-PAGE was performed using the procedure described by Reisinger and Eickacker (2006) with some modifications. The detail procedure is described in Part I of this dissertation. Similarly, Method 2, separation of different CP complex with PSI-LHCII and PSI-CP29 supercomplex were separated by BN-PAGE. Isolation of thylakoid and separation of different CP complexes were done according to Järvi et al. (2011).

2.6. Two dimensional polyacrylamide gel electrophoreses (2D-PAGE)

After the native BN-PAGE, each lane of the gel was excised and incubated for 30 min at room temperature in a denaturating buffer containing 125 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 2% beta-mercaptoethanol, and 10% glycerol. The denatured gel strip was loaded directly onto a 5% stacking gel for 2D-SDS-PAGE. The subunits of chlorophyll protein complexes were resolved on a 12% resolving gel. After electrophoreses at 80V for 2 h, proteins were transferred into the membrane. The membrane was incubated at 5% skim milk solution for 1 h. Finally, the membrane was incubated with CP29 antibody to detect CP29 protein.

2.7. Sucrose density gradient ultracentrifugation (SDGU)

Sucrose density gradient ultracentrifugation (SDGU) was performed to

isolate different chloroplast protein complex. PSI and PSII complexes were isolated by using SDGU according to (Dunahay and Staehelin, 1985). Similarly, 400 µg thylakoid membranes were used to isolate LHCII, PSI and PSII-LHCII supercomplex by using SDGU as described by (Tokutsu et al., 2012).

2.8. Immunoblotting analysis of sucrose density gradient ultracentrifugation (SDGU) sample

Each complex was carefully collected after sucrose density gradient centrifugation. Chlorophyll concentration was measured and aliquot of each complex that contained 2 µg chlorophyll were solublized in 25 mM Tris HCl (pH 6.8), 2% SDS, 6% glycerol, 1% β- mercaptoethanol and 0.004% bromophenol blue for 30 min at room temperature and polypeptides were separated by SDS-PAGE using 15% (w/v) acrylamide gel. Proteins were electrotransferred into Immobilon®-P Transfer membrane (Millipore). Immunoblotting has carried out by using specific antibodies. D1 antibody was used to detect D1 protein and phosphothreonine antibody was used to detect phosphorylation of PSII and LHCII proteins. In addition CP29 protein in each complex was detected by using CP29 antibody (Hwang et al., 2003).

2.9. Measurement of Low temperature chlorophyll fluorescence

Leaves segments were immediately frozen in liquid nitrogen after high light illumination. Fuorescence spectra of PSI and PSII were analyzed at 77K with liquid nitrogen by using F4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The emission spectra were recorded from 600-850 nm with excitation at 480 nm. Then PSI/PSII fluorescence ratio was calculated by

multiplying the fluorescence value of 732 nm (for PSI peak) by the value of 685 nm (for PSII peak).

2.10. Measurement of state transition in WT and *osstn8*

State transitions were measured according to Lunde et al. (2000) with some modifications using a pulse-amplitude-modulated fluorometer (PAM101/102/103, Walz Effeltrich, Germany). Dark adapted leaf discs were exposed to saturating white light for two seconds to determine maximal fluorescence (Fm), and subsequently illuminated for 15 min with 50 µmol m^{-2}s^{-1} blue light (BL) or 1,000 µmol m^{-2}s^{-1} high light (HL) to create state 2. The far-red light (FRL) was turned on to create state 1, and after 15 minutes the maximal fluorescence (Fm1) yield in state 1 was determined by giving the tissues a two second saturating white light pulse. The FRL was then switched off, the fluorescence was recorded with BL or HL for 15 min, and the maximum fluorescence yield in state 2 (Fm2) was then determined by giving a two second saturating white light pulse. The relative change in maximal fluorescence was calculated as, $\Delta F = (Fm1-Fm2) / Fm2 \times 100$ according to Bellafiore et al. (2005). Similarly, actinic high light (HL) at 1,000 µmol m^{-2}s^{-1} was used instead of blue light to measure state transition respectively.

2.11. Blue native gel electrophoreses (BN-PAGE) and immunoblotting of state 1 and state 2 leaves sample

Leaves sample were immediately collected after state 1 and state 2 conditions and immediately frozen in liquid nitrogen. The collection of leaves

were done each time period of illumination of state 2 and state 1 lights ie; blue light, high light, far red light respectively during the measurement of state transition with using PAM101/102/103. Thylakoid membranes were isolated and BN-PAGE has performed as described above. CP29 protein phosphorylation was analyzed by immunoblotting with CP29 antibody.

3. RESULTS

3.1. Phosphorylation of CP29 was found more in monocot plants

With a view to investigating whether phosphorylation of CP29 was general phenomenon in monocot plants (Chen et al., 2009), immunoblotting detection of phosphorylation of CP29 in docot plants such as Arabidopsis, spinach and cucumber was performed. As a result on contrary, high light illumination of these plants failed to phosphorylate CP29. The results show the strong phosphorylation of CP29 in WT rice after high light and light chilling (Fig 29A, B, C, D). In agreement with the results obtained from barley (Liu et al., 2009) about the phosphorylation of CP29 is general phenomenon in monocot plants rather than dicot.

3.2. Phosphorylation of CP29 was impaired in *osstn8* mutant plant

In Arabidopsis, STN8 kinase is required for the phosphorylation of PSII core proteins (D1/D2) and STN7 kinase is required for phosphorylation of LHCII (Bonardi et al., 2005, Vainonen et al., 2005), phosphorylation of CP29 in thylakoid membrane of both WT, *osstn7* and *osstn8* mutant leaves by immunoblot analysis using CP29 antibody has checked (Fig 29C, D, E). In the WT and *osstn7* leaves dark-adapted for 12 h, phosphorylation of CP29 was negligible, but phosphorylation of CP29 was observed by illumination of high light and light chilling. However the phosphorylation of CP29 was blocked in *osstn8* mutant. These results also reveal that phosphorylation of LHCII was observed at growth light and dephosphorylation of LHCII was observed at high light in both WT and
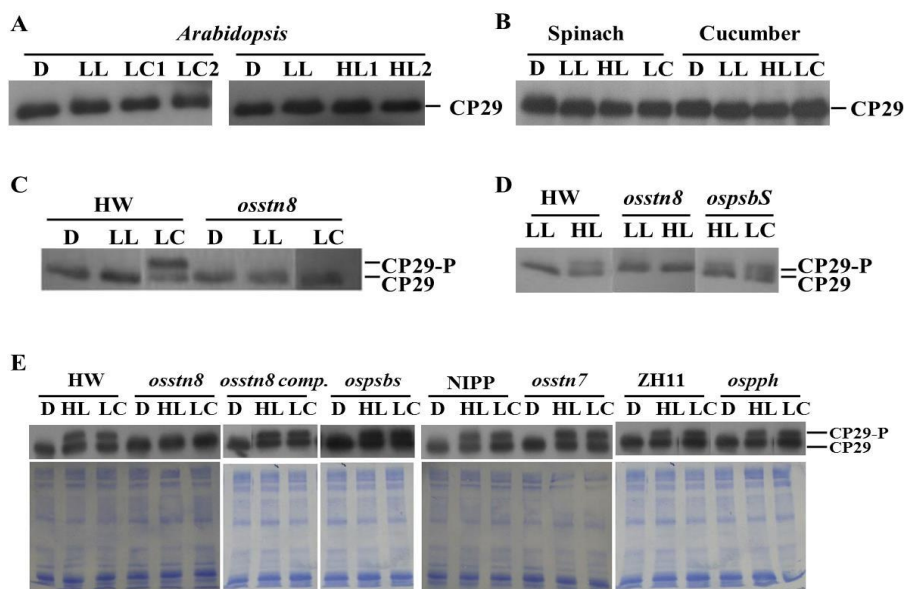
Figure 29. Difference in phosphorylation of CP29 in dicot and monocot plants. Thylakoid membranes were isolated after low light, high light and light chilling treatment and immunoblotting was done by using CP29 antibody to check the phosphorylation of CP29. (**A**) Analysis of CP29 protein phosphorylation in WT Arabidopsis, (**B**) Analysis of CP29 protein phosphorylation in spinach and cucumber, (**C, D**) Analysis of CP29 protein phosphorylation in rice WT, *osstn8* and *ospsbs* mutants, (**E**) CP29 protein phosphorylation in rice WT, *osstn8*, *osstn7* and *ospph* mutants. Following was the treatment condition in Arabidopsis, spinach and cucumber. D = Dark adapted for overnight, LL = Growth light 100 µmol m^{-2}s^{-1} for 2 h, LC1 = Light chilling for 12 h, LC2 = Light chilling for 25 h, HL1 and HL2 = High light illuminated leaf samples at 1,000 and 1,500 µmol m^{-2}s^{-1} for 2 h, HL = High light at 1,000 µmol m^{-2}s^{-1} for 2 h. In rice, D = Dark adapted leaves for overnight, LL = 200 µmol m^{-2}s^{-1} for 2h, LC = Light chilling for 15 h and HL = High light illuminated leaf samples at 1,500 µmol m^{-2}s^{-1} for 2 h. HW, NIPP and ZH11 are wild type of *osstn8*, *osstn7* and *ospph* mutants respectively.

osstn8 mutant plants (Part I, Fig 10A, B). However, CP29 phosphorylation was observed at high light illumination whereas D1 and CP43 protein were also phosphorylated. These data indicate that STN8 kinase is responsible for the phosphorylation of CP29 in rice.

3.3. PPH phosphatase dephosphorylates only LHCII but not CP29

In Arabidopsis, PPH phosphate has been identified for dephosphorylation of LHCII. To confirm this result, immunoblotting analysis by using phosphothreonine and CP29 antibody has used separately. Leaves were dark adapted to dephosphorylate all PSII and LHCII proteins then subsequently illuminated at low light and high light to phosphorylated LHCII and dephosphorylated LHCII. The result reveal that LHCII protein did not dephosphorylated in *ospph* mutant at different condition (Fig 31). This result supports the result of Samol et al. (2010) in Arabidopsis. Although CP29 dephosphorylation was observe in WT, *osstn7* and *ospph* after dark adaptation (Fig 31). Hence, it can conclude that there is no role of PPH phosphatase for dephosphorylation of CP29. In addition these data confirmed the role of STN7 kinase for LHCII phosphorylation and role of PPH phosphatase for LHCII dephosphorylation. Similarly the role of STN8 kinase for PSII core protein as well as CP29 protein phosphorylation and the role of PPH phosphatase was not for dephosphorylation of PSII core proteins and CP29 protein (Fig. 29E, Fig. 31). The photoinactivation of PSII was also more in *osstn7* and *ospph* mutants (Fig. 30).

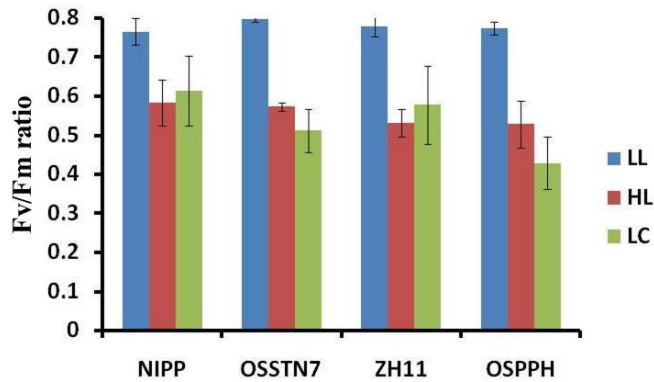## 3.4. STN7 kinase did not phosphorylate CP29 protein in rice

Figure 30. Photochemical efficiency of PSII during photoinhibition under Low light (LL), high light and light chilling. Fv/Fm ratio was calculated after high light treated leaf samples of WT, *osstn7* and *ospph* mutants after low light, high light and light chilling treatments. LL = low growth light at 200 µmol m^{-2}s^{-1} for 2 h, HL = 1,500 µmol m^{-2}s^{-1} for 1 and 2 h and LC = light chilling at 200 µmol m^{-2}s^{-1} for 15 h. Values are means ± SD of 5 replicates. NIPP and ZH11 are wild type of *osstn7* and *ospph* mutants respectively.

In Arabidopsis, STN7 kinase dependent phosphorylation of CP29 protein was observed (Fristedt and Vener, 2011; Tikkanen et al., 2006). However, it was hard to detect the CP29 phosphorylation in rice by immunoblotting with anti phosphothreonine antibody (Fig 31) and CP29 antibody respectively in Arabidopsis (Fig. 29A). Although it was easily detected the CP29 phosphorylation in WT rice (Fig. 29C, D, E). Similarly, CP29 phosphorylation was easily detected in WT and STN7 kinase mutant of rice (Betterle et al., 2015). The result suggests that phosphorylation of CP29 protein was observed in WT, *osstn7* and *ospph* but not in *osstn8* at high light illumination. This result further confirms the phosphorylation of CP29 is controlled by STN8 kinase in rice that is opposite result that was observed in Arabidopsis.

3.5. LHCII phosphorylation and dephosphorylation was impaired in *osstn7* and *ospph* mutants respectively

Phosphorylation and dephosphorylation of LHCII was analyzed in *osstn7* and *ospph* mutant. Therefore leaves were illuminated either with low light and high light or light chilling treatment. The result showed that phosphorylation of LHCII was blocked in *osstn7* mutant and dephosphorylation of LHCII was blocked in *ospph* mutant (Fig. 31). Although phosphorylation of PSII core proteins was similar with WT (Fig. 31).

3.6. State transition was impaired in *osstn7* and *ospph*

State transition can be detected as differential changes in fluorescence of
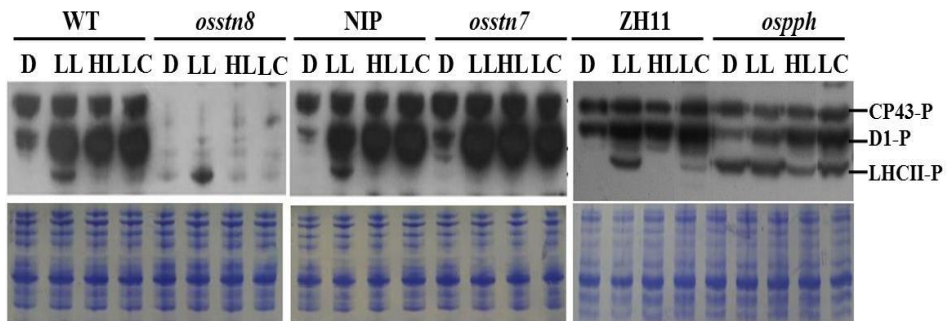
Figure 31. Analysis of phosphorylation of thylakoid membrane protein by immunoblotting with phosphothreonine antibody. Immunoblotting analysis of PSII and LHCII phosphorylation in WT, *osstn8*, *osstn7* and *ospph* mutant at different light illumination. The thylakoid membranes containing 2 µg chlorophyll was used in each lane. The SDS-PAGE after commassie blue stainingof the gel is shown in the bottom of each figure. D = dark adapted leaves sample, LL = low light at 200 µmol m^{-2}s^{-1} for 2 h, HL = high light at 1,500 µmol m^{-2}s^{-1} for 2 h and LC = light chilling at 200 µmol m^{-2}s^{-1} for 12 h. NIPP and ZH11 are wild type of *osstn7* and *ospph* mutants respectively.

PSII when leaves are exposed alternately to light favouring PSII and light favouring PSI. The relative change in maximal fluorescence during state transition, ΔF was 9.25 % in WT and 0 % in *osstn7* mutant in Fig. 32A and B respectively. Similarly, state transition was strongly impaired in *ospph* mutant because ΔF was 8.16 % in WT and 1.69 % in *ospph* mutant in Fig. 32C and D respectively. Therefore, these data confirm that the role of LHCII phosphorylation by STN7 kinase (Bellafiore et al., 2005) and LHCII dephosphorylation by PPH phosphatase (Shapiguzov et al., 2010) are required for state transition in rice.

3.7. The mobilization of PSII-LHCII supercomplex was suppressed in the *ospph* but not in *osstn7* mutant plants

In the blue-native polyacrylamide gel electrophoresis (BN-PAGE) experiment, the mobilization of PSII supercomplex was observed in WT and *osstn7* plants during HL illumination as well as light chilling treatment, and this process was significantly blocked in *ospph* mutants (Fig. 33A, B). Mobilization of PSII supercomplex was also blocked in *osstn8* mutant (Part I, Fig. 14A, B). Therefore, these results reveal that not only phosphorylation of PSII core protein but also dephosphorylation of LHCII was essential for mobilization of PSII supercomplex.

3.8. Low light/far red light induce state transition was observed in WT Arabidopsis but not observed at highlight/far red light

In WT Arabidopsis, LHCII phosphorylation was observed at low light
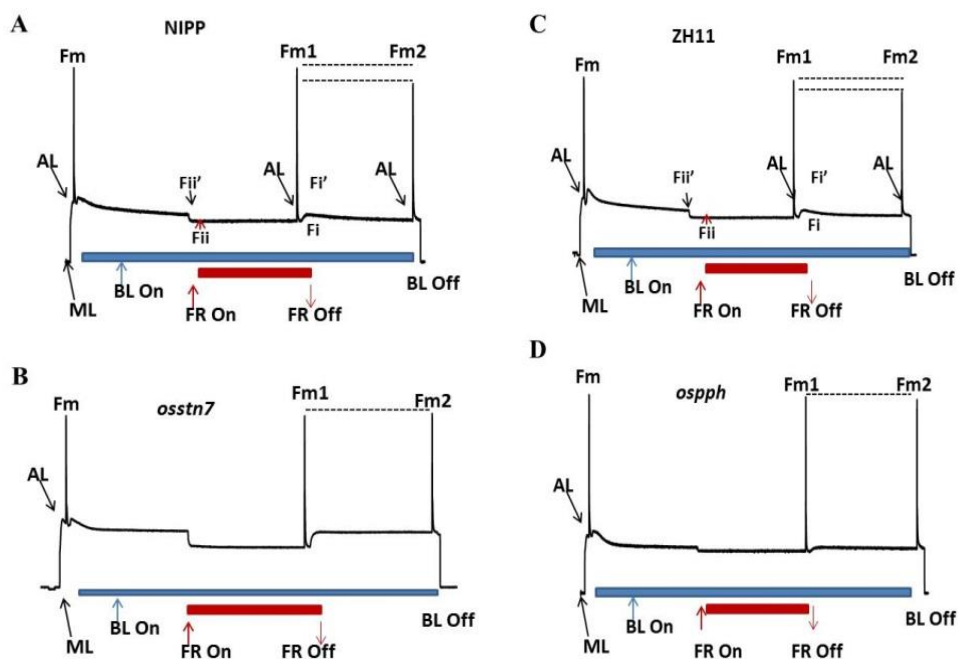
Figure 32. Measurement of state transition in WT, *osstn7* and *ospph* mutants. Measurement of state transition by using PSII light (blue light 20 µmol m^{-2}s^{-1}) and PSI light (far red light) in WT, *osstn7* and *ospph* mutants Uphead and down head arrows shows theturn on and turn off the respective light shown in figure. Far red light was illuminated for 15 min. (**A**) State transition in WT, (**B**) State transition in *osstn7*, (**C**) state transition in WT and (**D**) state transition of *ospph* mutant. ML = Measuring light, BL = Blue light AL = Actinic high light, FR = far red light, Fm, Fm1 and Fm2 was obtained by applying the saturated pulse at 4,000 µmol m^{-2}s^{-1} for 0.5 seconds. NIPP and ZH11 are wild type of *osstn7* and *ospph* mutants respectively.
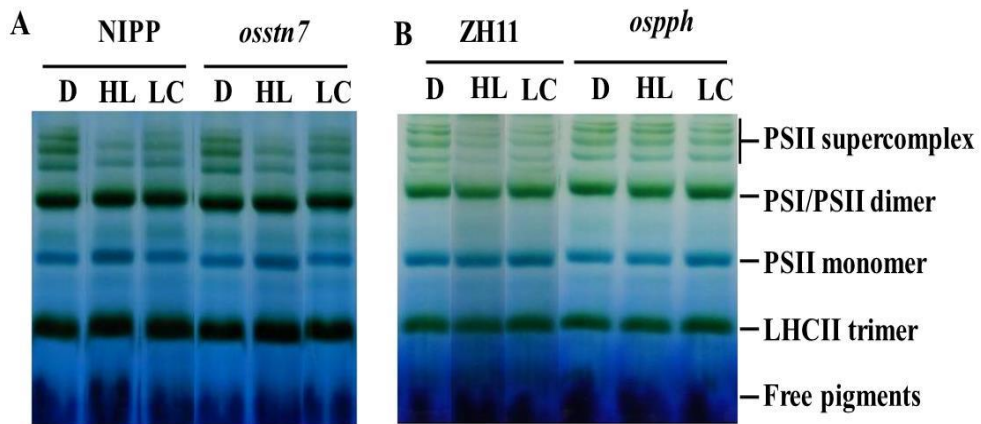
Figure 33. Separation of PSII-LHCII supercomplex by BN-PAGE. About 10 µg thylakoids from each plant's leaves were solubilized in n-dodycyl β-D-maltoside, and separated by 5-13.5 % BN-PAGE and different chloroplast protein (CP) complexes were separated. (**A**) BN-PAGE of rice WT and *osstn7* mutant, (**B**) BN-PAGE of rice WT and *ospph* mutant. D = dark adapted leaves sample, HL = high light at 1,500 µmol m^{-2}s^{-1} for 2 h and LC = light chilling at 200 µmol m^{-2}s^{-1} for 12 h.

but not at at high light (Part I, Fig 17B). In addition CP29 phosphorylation could not detected in Arabidopsis (Fig 29A). Therefore, state transition was measured in such condition where there was LHCII phosphorylation, without LHCII phosphorylation and with CP29 phosphorylation. The relative change in maximal fluorescence during state transition, ΔF was 7.3 % during blue light far red light and 0 % during high light far red light induce state transition (Fig. 34A, B). Therefore, result showed there was state transition when there was LHCII phosphorylation. However there was no state transition when there was no CP29 phosphorylation at HL.

3.9. PSI/PSII fluorescence ratio was increased in WT but not in *osstn8* at high light illumination

Low temperature chlorophyll fluorescence of PSI and PSII was measured and PSI/PSII fluorescence ratio was calculated. The data showed that low temperature chlorophyll fluorescence was similar in both WT and *osstn8* mutant leaves before HL illumination. However, PSI fluorescence increased more in the WT than in the *osstn8* mutant, the PSI/PSII fluorescence ratio (F686/F744) was more in WT and less in *osstn8* mutant (Fig. 35). This data support the role of CP29 phosphorylation to increased PSI fluorescence due to its mobilization from PSII to PSI during HL illumination.

3.10. State transition was impaired in *osstn8* mutant plant at HL illumination

State transition was measured in WT and *osstn8* by using actinic high light instead of low blue light. The actual state transition was altered between WT
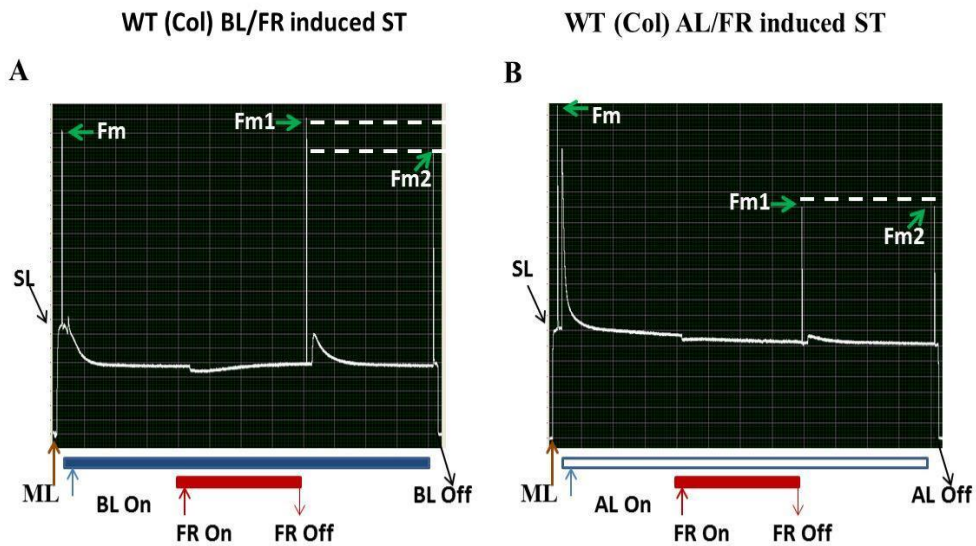
Figure 34. Measurement of state transition in WT Arabidopsis.
Measurement of state transitionby using PSII light either blue light 20 µmol m^{-2}s^{-1} or high actinic light and PSI light (far red light) in WT Arabidopsis. Uphead and down head arrows shows the turn on and turn off the respective light shown in figure. Far red light was illuminated for 15 min. (**A**) Low blue light far red light induced state transition, (**B**) Actinic high light and far red light induce state transition. ML = Measuring light, BL = Blue light AL = Actinic high light, FR = far red light, Fm, Fm1 and Fm2 was obtained by applying the saturated pulse at 4,000 µmol m^{-2}s^{-1} for 0.5 seconds.
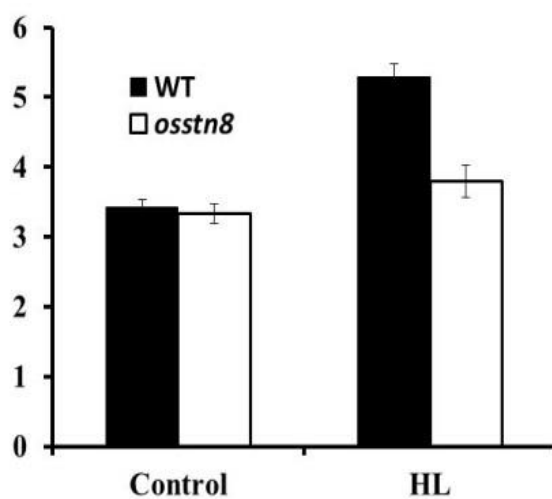
Figure 35. The 77K fluorescence emission spectra of WT and *osstn8* mutant leaves after high light illumination. 77K fluorescence emission spectra was recorded from 600-800 nm and PSI/PSII fluorescence was calculated by using the data of 732 nm emission for PSI and 685 nm emission for PSII. Control = dark adapted leaf segments and HL = high light illuminated leaf segments at 1,500 μmol m^{-2}s^{-1} for 2 h.

and *osstn8* when LHCII protein was dephosphorylated at high light (Fig. 31, 36) and CP29 protein was phosphorylated at high light illumination in rice and phosphorylation of CP29 was induced at high light illumination in WT but not *osstn8* mutant (Fig. 29C, D, E). The state transition was measured before and after the phosphorylation of CP29. The result reveal that the actual state transition was blocked in *osstn8* mutant plants as compared to WT before high light illumination (Fig. 36A, B, C, D) where CP29 was not phosphorylated but when the leaf were illuminated with high light to induce phosphorylation of CP29 the actual state transition was altered in *osstn8* mutant as compared to WT. Therefore this result show the phosphorylation of CP29 is essential for state transition and its phosphorylation is controlled by STN8 kinase.

3.11. First stage of state transition did not disassemble the PSII supercomplex at low light

State transition was also observed in WT and *osstn8* at blue light (state 2) and far red light (state 1). PSII supercomplex mobilization and immunoblotting analysis has performed to check the phosphorylation status of CP29 protein by using CP29 antibody. To confirm this result, leaf samples has collected from in each step during the measurement of state transition by PAM101/102/103. According to BN-PAGE, PSII supercomplexes were not mobilized at low light far red light induced state transition (Fig. 37A) and CP29 protein phosphorylation was not observed (Fig. 37D). In this stage the PSII supercomplexes that were not disassembled. Therefore, it is proposed that the model of first stage of state transition where PSII supercomplexes were not disassembled.
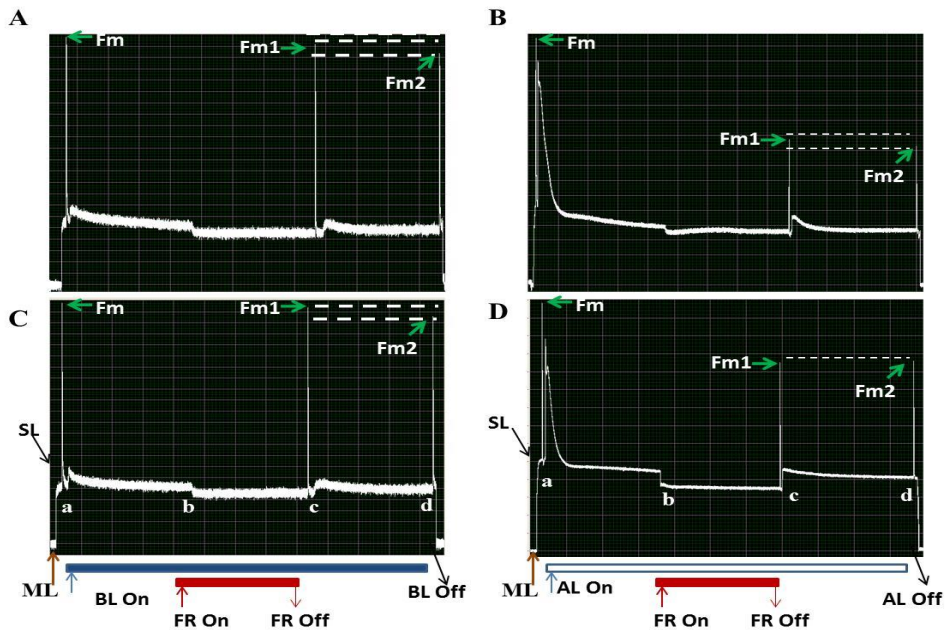
Figure 36. Measurement of state transition by using PSII light either blue light 20 µmol m^{-2}s^{-1} or high actinic light and PSI light (far red light) in WT and *osstn8* mutants of rice leaf. Uphead and down head arrows shows the turn on and turn off the respective light shown in figure. Far red light was illuminated for 15 min; low blue light or actinic light was also illuminated for 15 min during the time of measurement. (**A**) State transition in WT with blue light and far red light, (**B**) State transition in WT with high light and far red light, (**C**) State transition in *osstn8* mutant with blue light and far red light and (**D**) State transition in *osstn8* mutant with high light and far red light. ML = Measuring light, BL = Blue light AL = Actinic high light, FR = Far red light, Fm, Fm1 and Fm2 was obtained by applying the saturated pulse at 4,000 µmol m^{-2}s^{-1} for 0.5 seconds. Symbols "a" represent the dark condition, "b" represents the blue light or actinic light illumination time for 15 min, "c" represents the far red light illumination time 15 min and "d" represent again blue light or actinic light illumination time for 15 min.
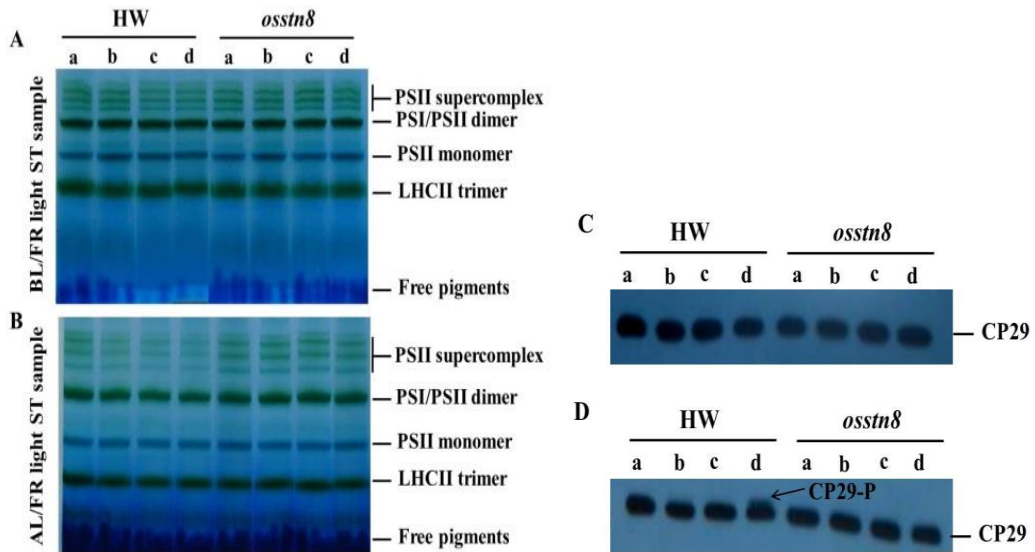
Figure 37. Analysis of the mobilization of PSII-LHCII supercomplex and phosphorylation of CP29 protein during state transition. Leaf samples were collected during the time of measurement of blue light; actinic high light or far red light induced state transition from Figure 36A-D. Thylakoid membranes were isolated and BN-PAGE was performed to separate different CP complexes including PSII-LHCII supercomplex. (**A**) BN-PAGE of WT and *osstn8* mutant during low blue light and far red light induced state transition, (**B**) BN-PAGE of WT and *osstn8* mutant during actinic high light and far red light induced state transition, (**C, D**) Immunoblotting assay of thylakoid membranes to detect CP29 protein phosphorylation as in Fig A and B. Symbols "a" represent the dark condition, "b" represents the blue light or actinic light illumination time for 15 min, "c" represents the far red light illumination time 15 min and "d" represent again blue light or actinic light illumination time for 15 min.

3.12. Second stage of state transition disassembled the PSII supercomplex at high light

Previous research has identified the role of CP29 protein phosphorylation for state transition in Arabidopsis (Tikkanen et al., 2006; Fristedt and Vener 2011), and state transition in *Chlamydomonas* (Tokutsu et al., 2009). To confirm this result, leaf segments of WT and *osstn8* were illuminated at high light. It is found that PSII supercomplex mobilization was impaired in *osstn8* where CP29 phosphorylation was blocked at high light illumination. To confirm this result, again leaf samples were collected in each step during the measurement of state transition by PAM101/102/103. Immunoblotting result showed the phosphorylation of CP29 protein was observed at state 2 (white high light) in WT but not in *osstn8* mutant where PSII supercomplex were mobilized in WT but not in *osstn8* (Fig. 37B, D). Therefore based on this result, this stage was name as second stage of state transition that was controlled by CP29 protein phosphorylation.

3.13. Phosphorylation of LHCII was observed at low light but not high light and light chilling treatment

LHCII protein phosphorylation kinetics has studied and it was observed that LHCII phosphorylate only at low light but not at high light. Even short period of high light illumination and long period of light chilling does not phosphorylates LHCII protein (Fig. 38A, B). Therefore according to result, the phosphorylation of LHCII was only observed at low light illumination.

3.14. High light induce phosphorylation of CP29 form the PSI-LHCI-CP29 supercomplex

To further examine the association of CP29 in PSI, BN-PAGE of thylakoid membranes from WT and *osstn8* mutant after dark and high light illumination. First PSI and PSII complex were isolated by SDGU in WT and*osstn8* mutant (Fig. 39A). Each complex of PSI and PSII were used for immunoblotting with CP29 and phosphothreonine antibody. According to Fig. 39B, one phospho protein was appeared in PSI only in WT and that was absent in *osstn8* mutant. The phosphoprotein that has similar size with phospho D1 protein may be the phosphor CP29 protein. Similarly, immunoblotting with CP29 antibody has performed in the same sample and found that CP29 was detected in the PSI complex in WT but not in *osstn8* after high light illumination (Fig. 39C). Similar result was obtained in isolated complex of LHCII, PSI and PSII-LHCII supercomplex that was obtained by SDGU (Fig 40A, C, D).

To check the mobilization of CP29 from PSII supercomplex to PSI/PSII dimer, 2D-PAGE of BN-gel has performed and immunoblotting was carried by using CP29 antibody. The data showed CP29 was detected in PSI complex of BN-gel after high light illumination in WT. The 2D result show the presence of extra bands in PSI complex of WT but not in *osstn8* mutant and this extra band was disappeared in dark adapted leaves in WT (Fig 41A, B). This data clearly shows that phosphorylation of CP29 is found during high light illumination which causes its dissociation from PSII and reassociation into PSI during high light illumination.

3.15. Low light induce phosphorylation of LHCII form the PSI-LHCI-LHCII complex and high light induce phosphorylation of CP29 form PSI-CP29 complex

Formation of PSI-LHCII and PSI-CP29 complex during low light and high light illumination has observed in WT leaf. According to BN-PAGE result, PSI-LHCII complex was observed at low light illuminated leaf sample and PSI-CP29 complex was observed only at high light illuminated leaf sample (Fig. 42). This result conclude that both major and minor light harvesting complex proteins LHCII and CP29 are phosphorylates at low light and high light and moves into PSI for state transition.
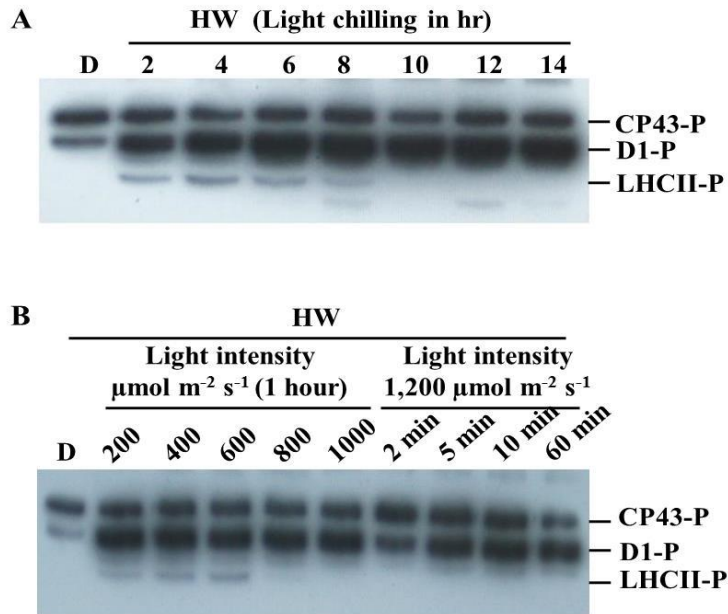
Figure 38. Analysis of phosphorylation of LHCII protein in WT rice at different environmental condition. After dark adapted, leaf segments from WT rice were treated with different environmental condition. Thylakoid membranes were isolated and phosphorylation of LHCII protein was detected by using phosphothreonine antibody. (**A**) LHCII protein phosphorylation in WT rice during light chilling treatment at 4°C for 2 to 14 h and (**B**) LHCII protein phosphorylation during low light and high light illumination.
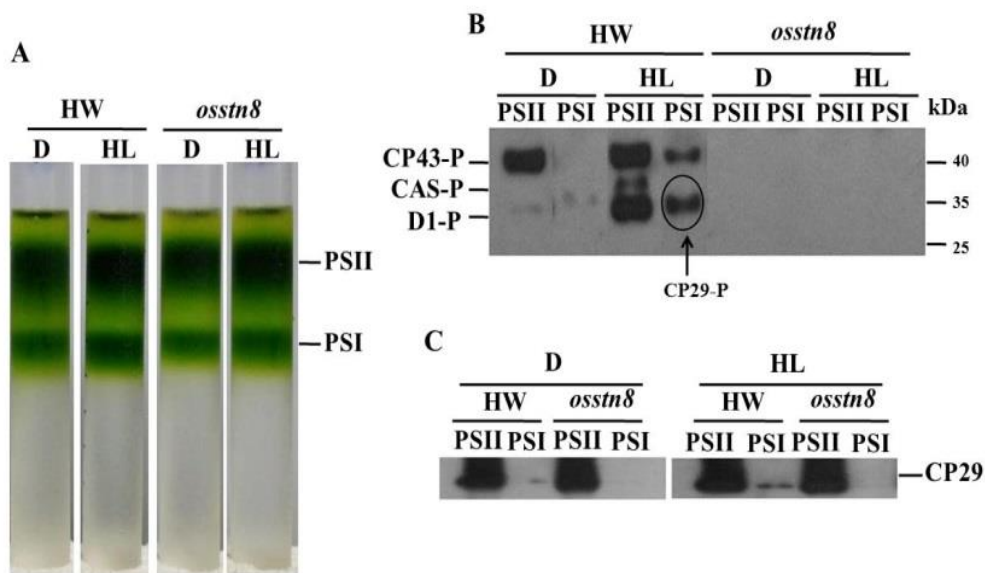
Figure 39. Separation of PSI and PSII complexes by sucrose density gradient ultracentrifugation (SDGU) in WT and *osstn8* mutant and immunoblotting assay. (**A**) Isolation of PSI and PSII complex in WT and *osstn8* mutant. Thylakoid membranes isolated from WT and *osstn8* mutant plants were subjected to sucrose density gradient ultracentrifugation. D, dark-adapted leaf segments for 12 h; HL, high light illuminated leaf samples at 1,500 µmol m^{-2}s^{-1} for 2 h, (**B**) PSI and PSII complexes were subjected to immunoblotting to check the phosphorylation of PSII proteins. Phosphothreonine antibody was used to detect several phosphoproteins, (**C**) PSI and PSII complexes were subjected to immunoblotting to check the CP29 protein by using CP29 antibody. Aliquot of isolated PSI and PSII complexes containing 2 µg chlorophylls was used.
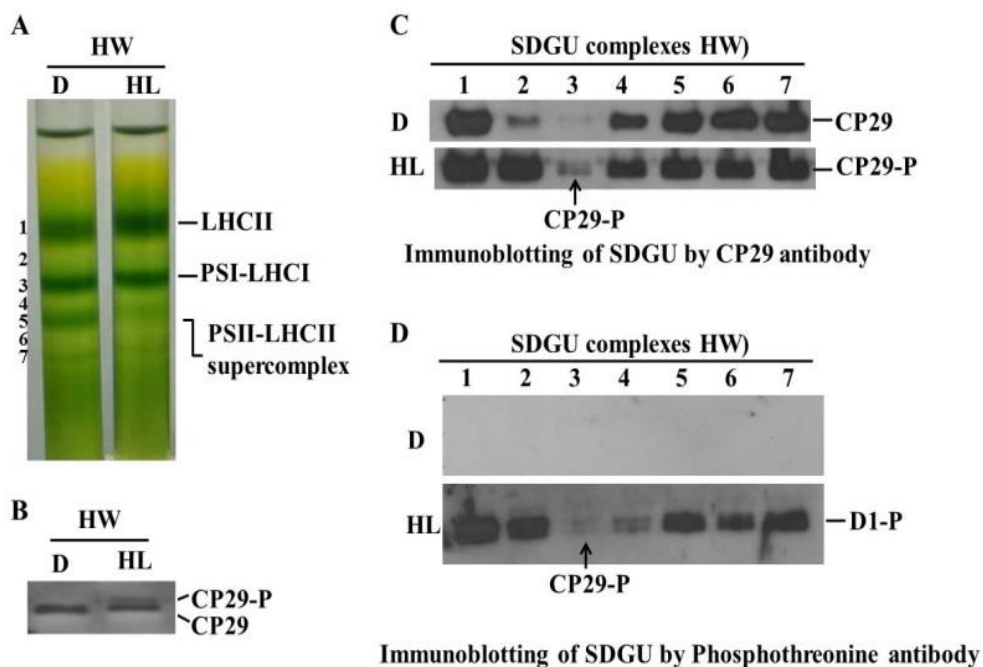
Figure 40. Separation of LHCII, PSI and PSII-LHCII supercomplex by sucrose density gradient ultracentrifugation (SDGU) in WT and observation of CP29 mobilization into PSI during high light illumination. (**A**) Isolation of LHCII, PSI and PSII-LHCII supercomplex in WT rice leaf samples.Thylakoid membranes isolated from WTmutant plants were subjected to sucrose density gradient ultracentrifugation. D, dark-adaptedleaf segments for 12 h; HL= high light illuminated leaf samples at 1,500 µmol m^{-2}s^{-1} for 2 h. (**B**) Phosphorylation status of CP29 protein was checked in same thylakoid membranes that was subjected to SDGU, (**C**) LHCII, PSI and PSII-LHCII supercomplex were subjected to immunoblotting to check the phosphorylation of PSII proteins by using Phosphothreonine antibody, (**D**) LHCII, PSI and PSII-LHCII supercomplex was subjected to immunoblotting to check the CP29 protein by using CP29 antibody. Aliquot of isolated LHCII, PSI and PSII-LHCII supercomplex containing 2 µg chlorophylls was loaded in each lane.
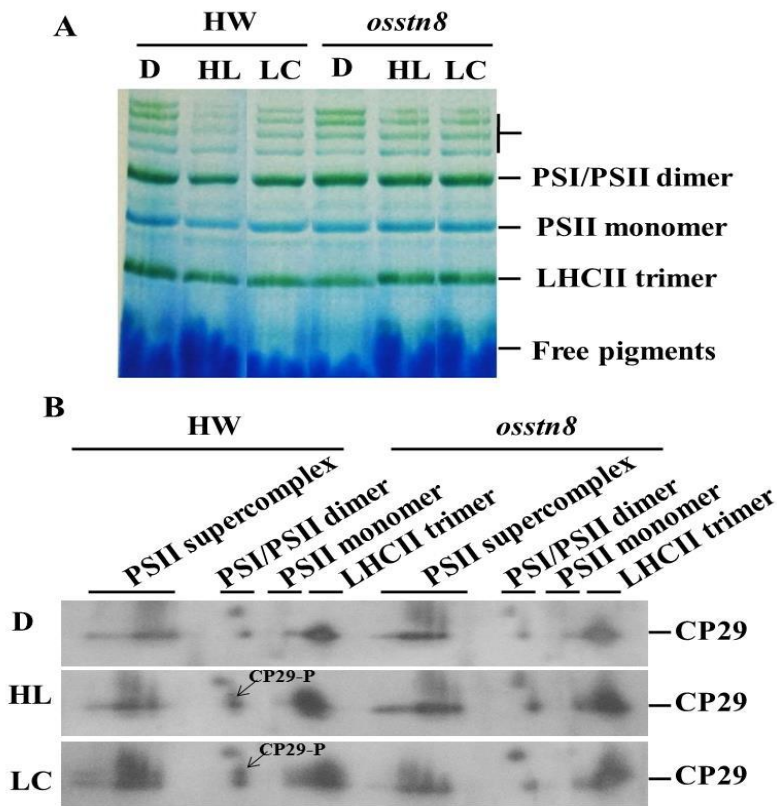
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Figure 41. Separation of PSII-LHCII supercomplex by BN-PAGE and analysis of CP29 mobilization into PSI with two dimensional electrophoreses during high light illumination. About 10 µg thylakoids from each plant's leaves were solubilized in n-dodycyl β-D-maltoside, and separated by 5-13.5 % BN-PAGE and different chloroplast protein (CP) complexes were separated. (**A**) BN-PAGE of rice WT and *osstn8* mutant, (**B**) two dimensional immunoblotting of BN-PAGE was conducted and immunoblotting with CP29 antibody. D = dark adapted leaves sample, HL = high light at 1,500 µmol m^{-2}s^{-1} for 2 h and LC = light chilling at 200 µmol m^{-2}s^{-1} for 12 h.
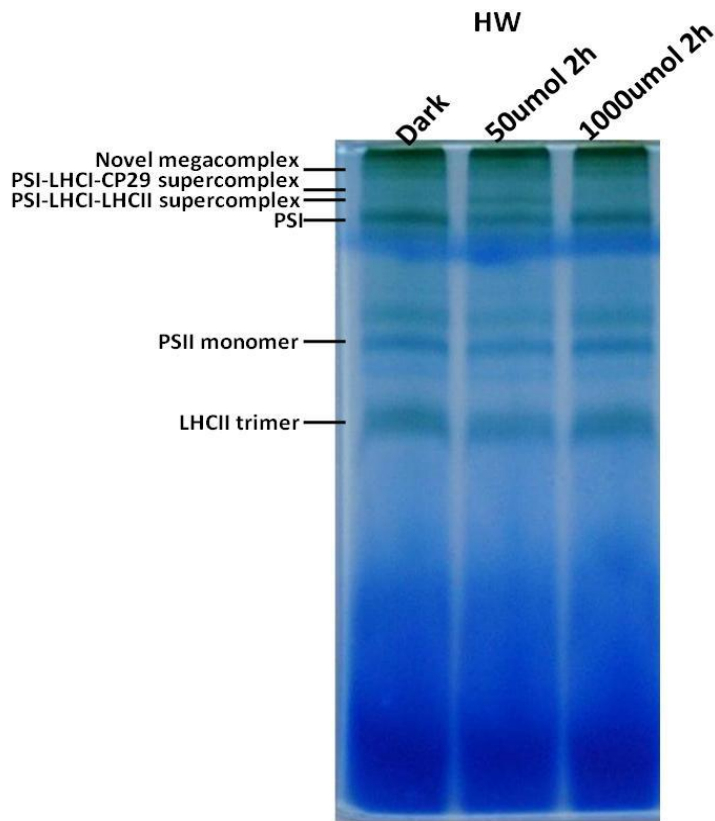
Figure 42. Separation of different chloroplast protein (CP) complexes PSI-LHCI-LHCII supercomplex and PSI-LHCI-CP29 supercomplex by BN-PAGE during low light and high light illumination. An aliquot of thylakoid membranes that contain About 10 µg chlorophyll from each plant's were solubilized in n-dodycyl β-D-maltoside, and separated by 5-13.5 % BN-PAGE and different chloroplast protein (CP) complexes were separated. BN-PAGE of rice WT and *osstn8* mutant, D = dark adapted leaves sample, LL = low light at 50 µmol m^{-2}s^{-1} for 1 and 2 h, HL = High light at 1,500 µmol m^{-2}s^{-1} for 1 and 2 h, and LC = light chilling at 200 µmol m^{-2}s^{-1} for 12 h.

4. DISCUSSION

Previously, it has been observed that the CP29 protein was phosphorylated in C3 (barley) and C4 (maize) plants during cold treatment (Bergantino et al., 1995; Bergantino et al., 1998) and CP29 phosphorylation was weakly detected in Arabidopsis during PSII light illumination (Tikkanen et al., 2006) and high light illumination by STN7 kinase (Fristedt and Vener, 2011). Later Chen et al. (2013) proposed the phosphorylation of CP29 is widespread phenomenon in monocot plants but not in dicot plants. Hence, based on above controversies the phosphorylation of CP29 protein in Arabidopsis, spinach, and cucumber as docot model plants as well as rice as a monocot model plants at different environmental condition has checked. Interestingly the result clearly demonstrates the phosphorylation of CP29 was only observed in monocotyledons plant (rice) during high light illumination and light chilling treatment. However CP29 phosphorylation was not observed in dicot plants such as Arabidopsis, cucumber and spinach during high light illumination and light chilling treatment (Fig. 29). Recently, Betterle et al. (2015) observed the phosphorylation residue of CP29 between dicot and monocot model plants rice and Arabidopsis. According to their result CP29 was phosphorylated at Thr-82 in the sequence of mature CP29 protein in rice but they could not observed CP29 phosphorylation at Thr-112 or Thr-114 that were previously identified as phosphorylation site in dicot Arabidopsis at high light treatment (Betterle et al., 2015). Hence this result clearly suggest that rapid phosphorylation of CP29 is a general phenomenon in monocots, but not in dicots under highlight and light chilling treatment.

In recent year two major kinases has been identified in the the thylakoid membrane proteins of higher plants. STN8 kinase has identified for PSII core

protein phosphorylation and STN7 kinase has identified for LHCII protein phosphorylation (Bonardi et al., 2005; Nath et al., 2013b; Betterle et al., 2015). Reversible phosphorylation of major PSII-LHCII proteins and their substrates was known although reversible phosphorylation of minor light harvesting complex II, CP29 and its kinase and phosphatase was still controversial. Hence, in this dissertation, a kinase that is specific for CP29 protein phosphorylation has identified. These data clearly shows the CP29 phosphorylation was blocked in *osstn8* mutant but not in *osstn7* and *ospph* mutant, and recovery of CP29 phosphorylation was observed in *osstn8* complement plant during high light illumination. These results verify the role of STN8 kinase not only phosphorylates PSII core protein (D1, D2 and CP43) but also phosphorylates minor LHCII protein CP29. It has been postulated that lacks of NPQ deactivates STN7 kinase (Tikkanen et al., 2010), hence CP29 phosphorylation in *osPsbS* mutant of rice was checked. Interestingly CP29 phosphorylation was observed in *osPsbS* mutant during high light illumination. This result further confirm the STN8 kinase in rice regulates CP29 phosphorylation since inactivation of STN7 kinase did not blocked CP29 phosphorylation. Similarly two phosphatases for dephosphorylation of LHCII protein by PPH1 and TAP38 was previously identified (Shapiguzov et al., 2010; Pribil et al., 2010). Recently photosystem II core phosphatase (PBCP) has identified for dephosphorylation of PSII core proteins (Samol et al., 2012) that facilitate the mobilization of damaged PSII from grana to stroma lamellae for PSII repair (Vainonen et al., 2005; Tikkanen et al., 2008). To verify this result, dephosphorylation assay of CP29 protein by activating phosphatase in the dark has performed. Interestingly, PSII core proteins, LHCII and CP29 proteins dephosphorylated at dark in *osstn7*, *osstn8* and *ospph* mutants. It has been noted that CP29 protein dephosphorylated in *ospph* mutant that suggest the role of PPH phosphatase only for dephosphorylation of LHCII

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but not CP29 because dephosphorylation of LHCII was blocked in *ospph* mutant. Hence kinase and phosphatase for LHCII and CP29 might be different to each other although both of them are light harvesting complex proteins. Therefore STN8 kinase is suitable candidate for CP29 phosphorylation in monocot plants rice. At the present, the phosphatase of CP29 is still unknown and probably PBCP phosphatase might be the suitable candidate to dephosphorylate CP29 although PBCP phosphatase mutant of rice has not identified yet.

The low blue light and far red induced state transition was similar between WT and *osstn8* where only LHCII was phosphorylated. Similarly, the data of BN-PAGE shows mobilization of supercomplex could not observed during blue light and far red light induced state transition in WT and *osstn8* mutant. It means PSII supercomplex could not mobilized when LHCII protein was phosphorylated during state transition. This data further support the result obtained in Arabidopsis by Wientjes et al. (2013a). However, state transition was impaired in *osstn7* and *ospph* mutants where LHCII phosphorylation and dephosphorylation was blocked. Therefore, the reversible phosphorylation of LHCII protein was required for state transition.

Several theories has been proposed about state transition however, a question arose whether the first step of high light induced mobilization is the same as the first step of PSII light induced state transition, or not?. The mobilization of PSII-LHCII supercomplex was observed before the start of PSII repair during high light illumination, which involved the phosphorylation of PSII core proteins including CP43. In the second step of the state transition to State 2 the actual mobilization of PSII supercomplex is initiated by the phosphorylation of LHCII by STN7, which decouples LHCII from the supercomplex to be migrated to PSI. However, LHCII is known to be phosphorylated and

dephosphorylated very rapidly in the beginning of high light illumination. In the agreement of Galka et al. (2012), LHCII serves as an antenna of both PSI and PSII in most natural light conditions to achieve excitation balance between the two photosystems (Wientjes et al., 2013b). Through the time-resolved fluorescence measurements on the photosynthetic thylakoid membranes they could show that LHCII even became more efficient light harvester when it was associated with PSI than with PSII (Wientjes et al., 2013b). In *Chlamydomonas reinhardtii* PSI-LHCI supercomplex strongly associated with CP29 was isolated and this complex help binding with LHCII in State 2 (Kargul et al., 2005; Kargul and Barber, 2008; Tokutsu et al., 2009). Phosphorylation of Lhcb proteins was important for the regulation of state transitions (Depège et al., 2003; Pesaresi et al., 2009) and CP29 was known to be strongly phosphorylated during high light illumination and under stress conditions including chilling in the light (Hwang et al., 2003; Chen et al., 2013) .

This research work has investigated the important aspects of state transition in rice plants at the level of PSII protein phosphorylation and supercomplex mobilization. However, note that other mechanisms, such as thylakoid membrane reorganization also occur during state transitions (Wientjes et al., 2013a). To test the possible role of phosphorylation of LHCII and CP29 in WT and mutant, state transition was measured before and after high light illumination (before and after phosphorylation of LHCII and CP29) was measured. The result reveals that the actual state transition was similar in WT and *osstn8* mutant however when the leaves were illuminated with high light, the actual state transition was impaired in *osstn8* but not in WT. To confirm this result low temperature chlorophyll fluorescence before and after high light illumination was measured. The data show the increased in PSI/PSII fluorescence ratio only in WT but not in *osstn8* mutant. When leaves were illuminated with

high light there were only PSII core protein and CP29 protein were phosphorylated and but not LHCII protein. Hence 77K fluorescence data show the additional role of CP29 phosphorylation for state transition during high light illumination. Hence based on above observation, two types of state transition have been proposed ie; first stage of state transition [ST1] and second stage of state transition [ST2] that is discussed below:

In low light state transition was observed in *osstn8* mutant when there was only LHCII protein was phosphorylated without PSII supercomplex mobilization. Hence PSII core protein phosphorylation was not required for state transition and PSII supercomplex bwas not mobilized at low light. In addition PSII core protein and CP29 protein phosphorylation was observed in WT but not in *osstn8* mutant. This data suggest that both PSII core protein and CP29 protein are required for PSII supercomplex mobilization. In addition, when leaves were illuminated under high light, PSII core protein and CP29 protein are phosphorylated and the dephosphorylation of LHCII causes PSII supercomplex mobilization and phosphorylated CP29 moves to PSI. In *ospph* mutant PSII supercomplex mobilization was slower therefore dephosphorylation of LHCII was required as in high light illumination in WT. For further evidence, during high light illumination, PSII supercomplex mobilization was observed in *osstn7* mutant where LHCII phosphorylation was blocked. Hence only LHCII phosphorylation was not required for mobilization of PSII supercomplex. Therefore based on this current research two stages of state transition ie; ST1 and ST2 has been proposed. In ST1 phosphorylated LHCII moved to PSI without PSII supercomplex mobilization and in ST2 phosphorylated CP29 moves to PSI with PSII supercomplex mobilization in rice plants. In rice plant it is hard to predict the role of PSII core protein phosphorylation in ST2. Therefore at the end, it is proposed the PSII supercomplex mobilization induce PSII repair and state transition distribute

energy between PSI and PSII and PSII supercomplex mobilization in high light was for both PSII repair and energy distribution in ST2.

To look high light induced phosphorylation dependent CP29 dissociation from PSII supercomplex different chloroplast protein (CP) complex including PSII supercomplex, PSI complex etc were isolated either by sucrose density gradient ultracentrifugation (SDGU) or by BN-PAGE in WT and *osstn8* mutant after highlight illuminated leaf samples. Immunoblotting assay by CP29 antibody has performed in each complex isolated by SDGU. The distribution of CP29 protein in PSI was observed in WT but not in *osstn8* mutant during high light illumination. This result confirms the mobilization of CP29 from PSII supercomplex to PSI is phosphorylation dependent by STN8 kinase. Similarly phosphorylation dependent mobilization of CP29 into PSI was observed in *Chlamydomonas reinhardtii* during state 2 conditions (Takahashi et al., 2006; Tokutsu et al., 2009). According to Fristedt and Vener, 2011 the relocation of CP29 from PSII supercomplex was phosphorylation dependent by STN7 kinase in Arabidopsis. Later Betterle et al., 2015 verified that the phosphorylation of CP29 was not controlled by STN7 kinase in rice and they could not observe the CP29 in stroma lamellae during high light illumination. However some CP29 was found in PSI during high light illumination in WT but not in *osstn8*. The difference in the result might be due to isolation procedure and detergent used.

For further proof that supports the mobilization of phospho CP29 into PSI by STN8 kinase, different CP-complexes were separated by BN-PAGE. Interestingly, it was observed PSI-LHCII complex when the leaf were illuminated in low light where LHCII was phosphorylated. The result was similar with phosphorylation of LHCII at low light and its mobilization into PSI (Järvi et al., 2011). Interestingly PSI-CP29 complex was observed only in WT during high

light and light chilling treatment only in WT but not in *osstn8* mutant. Hence PSI-CP29 complex was only observed where CP29 was phosphorylated. This result suggests the phosphorylation dependent mobilization of CP29 into PSI is controlled by STN8 kinase in rice. Again when leaves were switch from high light to low light then dephosphorylation and relocation of CP29 from PSI to PSII supercomplex was observed. In addition, 77K chlorophyll fluorescence was measured in WT and *osstn8* mutant's leaf before and after high light illumination. These data shows the increment of PSI/PSII fluorescence ratio in WT but not in *osstn8* mutant after high light illumination. This result also support the increment of PSI antenna size was due to mobilization of CP29 at high light illumination.

In overall, this research work clearly suggest the role of STN8 kinase in rice not only for the phosphorylation of PSII core proteins but also for phosphorylation of minor light harvesting complex protein, a CP29 during high light illumination. These data by using *osstn8* complement plant show the core kinase for CP29 phosphorylation is STN8 kinase but not STN7 kinase. In this study, it is shown that the movement of phosphorylated CP29 at high light and phosphorylated LHCII at low light shows state transition. Therefore, it has given two new names for the process of state transition ie state transition 1 [ST1] and state transition 2 [ST2].

Finally it is concluded that the process of ST1 occur when LHCII become phosphorylated and move to PSI although PSII supercomplex does not mobilize. However in ST2 the CP29 proteins become phosphorylates and moves to PSI where PSII supercomplex was mobilized. These two processes of state transition are regulated by LHCII phosphorylation by STN8 kinase in low light and CP29 phosphorylation by STN8 kinase at high light in rice plants (Fig. 43).
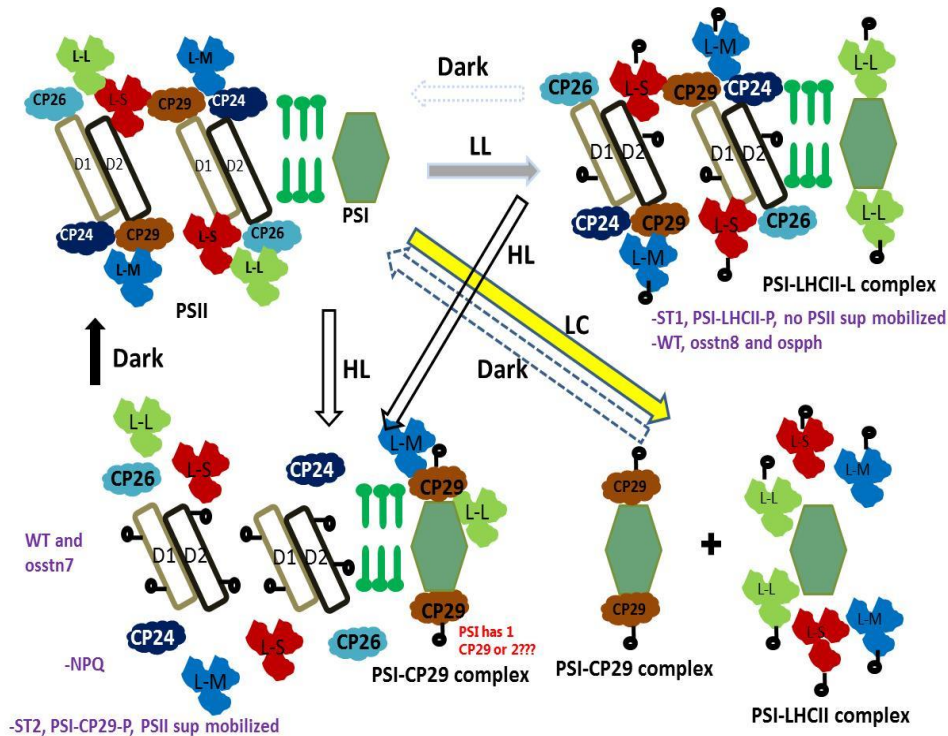
Figure 43. Schematic representations of mobilization of PSII supercomplex and phosphorylation of PSII proteins sequential events during state transition. In fully functional PSII complex, LHCII is phosphorylates at LL and it form PSI-LHCII complex without PSII supercomplex mobilization at state transition 1 (ST1) and CP29 is phosphorylates and form PSI-CP29 complex during high light illumination to state transition 2 (ST2). Although the mobilization of PSII supercomplex is required for state transition is not identified yet.

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벼의 상태 전이 및 광계 II 초복합체의 이동 과정 동안 틸라코이드 단백질 인산화 과정의 조절

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요약

애기장대를 이용하여 얻은 최신 연구결과들을 보면, STN8 인산화 효소는 D1, D2, CP43, PsbH, TSP9, CaS 등의 광계 II 핵심 단백질들을 인산화하고 STN7 인산화 효소는 광수확복합체 II (LHC II) 단백질뿐만 아니라 광계 II 핵심 단백질들을 인산화하고 상태 전이 현상에 참여한다는 것을 알 수 있다. 하지만 STN7 및 STN8 인산화 효소 둘 다 불활성화 된 애기장대 돌연변이 개체에서만 광계 II 핵심 단백질의 인산화 과정이 완전히 차단되는 것이 관찰된다. 이러한 이유로 STN8, STN7 그리고 PPH 유전자에 T-DNA를 삽입하여 만든 결손 돌연변이 벼 개체 (*Oryza sativa* L.)인 *osstn8*, *osstn7* 그리고 *ospph* 를 이용하여 STN8 및 STN7 인산화 효소의 특정 역할을 설명해왔다. 해당 연구 결과에서는 *osstn8*, *osstn7* 돌연변이 벼 개체를 이용하여 광계 II 수리 과정 조절, 광계 II 초복합체의 이동 및 상태 전이 현상이 일어나는 동안에 발생하는

광계 II – 광수확복합체 II 단백질 복합체의 인산화 과정의 역할을 명확하게 제시해 왔다.

야생형 개체와 비교했을 시, STN8 돌연변이 개체(*osstn8*)에서 광계 II 핵심 단백질 인산화 과정(PCPP)이 확실히 감소하였고, 그라나는 얇고 길게 늘어진 형태를 띠었다. 그리고 고광(HL) 스트레스에 의한 광계 II 불활성화 정도는 야생형보다 심하였고, 손상된 D1 단백질의 분해는 야생형에 비해 빠르지 않았다. 또한 수리 기작인 그라나에서 스트로마로의 광계 II 초복합체 이동 과정도 야생형에 비해 감소하였다. 이러한 결과들은 STN8 유전자의 부재로 인해 STN8이 결손된 벼 돌연변이 개체에서 광계 II 핵심 단백질 인산화 과정(PCPP)이 원활하게 일어나지 않는다는 것과 동시에 광계 II 수리 기작에서의 STN8 유전자의 중요성을 증명하고 있다. 그리고 이런 동일한 모습들이 애기장대의 이중 돌연변이 개체에서도 보이고 있다.

고광 스트레스에 의한 과산화 음이온 라디칼 및 과산화수소의 발생 정도는 STN8 돌연변이 개체가 더 높았다. 또한 과산화 음이온 라디칼의 디스뮤테이즈가 활성화 되지 않을 때, 과산화 음이온 라디칼의 발생 정도는 증가하였다. 이는 과산화 음이온 라디칼의 생성 과정이 과산화수소의 생성 과정보다 더 앞서 발생한다는 것을 보여준다. 하지만 야생형과 STN8 돌연변이 개체 간의 단일 산소 발생 정도는 차이점이 없었다. 게다가 광계 II에서의 과산화 음이온 라디칼 발생 정도는 야생형에 비해 STN8 돌연변이 개체에서 추출한 광계 II 및 광계 II – 광수확복합체 II 단백질 복합체 그리고 틸라코이드 막에서 더 높았다. 또한 고광 스트레스에 의한 단백질의 산화 정도는 STN8 돌연변이 개체에서 추출한 광계 II 및 광계 II – 광수확복합체 II 단백질 복합체에서 더 높았다. 이러한 결과는 과산화 음이온 라디칼이 STN8 돌연변이 개체에서 발생하는 활성 산소의 주요 형태이며, 고광 스트레스에 의해 초복합체에서 손상된 광계 II는 과산화 음이온 라디칼이 발생하는데 있어 초기에 기여한다는 것을 보여준다.

이외에, CP29 단백질에 대한 실험 또한 진행하였다. CP29 단백질의 인산화 효소 및 탈인산화 효소는 여전히 알려져 있지 않다. 이러한 이유로 PPH 탈인산화 유전자 및 STN7, STN8 유전자에 T-DNA를 삽입한 벼 돌연변이 개체를 이용하여 해당 효소들에 대해 알아보려 하였다.

고광 스트레스 및 저온 스트레스에 의해 발생한 CP29의 인산화 형태는 야생형, STN8 돌연변이의 보완 개체, STN7 돌연변이 그리고 PPH 돌연변이 개체에서 관찰되었으나 STN8 돌연변이 개체에서는 관찰되지 않았다. 하지만 암처리를 진행한 후에는 CP29의 탈인산화 형태가 모든 개체에서 관찰되었다.

광수확복합체 II의 인산화 및 탈인산화 형태의 경우 STN7 돌연변이 개체 및 PPH 돌연변이 개체에서 관찰되지 않았고 이에 의해 상태 전이 현상도 감소하였다. 그리고 고광 스트레스에 의한 광계 II 초복합체의 이동 현상은 STN7 돌연변이 개체에서 관찰되었으나 PPH 돌연변이 개체에서는 감소하였다. 이러한 결과들은 광수확복합체 II 단백질의 탈인산화가 광계 II 초복합체의 이동 현상에 수반된다는 것을 제시하고 있다.

보통의 조건에서, 상태 전이 현상 동안 발생하는 최대 형광의 상대적 변화인 ΔF는 야생형과 STN8 돌연변이 개체 간에 차이점이 없었고 광계 II 초복합체의 이동 또한 관찰되지 않았다. 하지만 고광 스트레스 처리 후 ΔF는 야생형에 비해 STN8 돌연변이 개체에서 확실하게 줄어들었고 광계 II 초복합체의 이동은 야생형에서만 관찰되었다. 또한 고광 스트레스 조건 하에 CP29의 인산화 형태는 야생형의 광계 I 복합체에서만 관찰되었다. 그러므로 고광 스트레스에 의한 CP29의 인산화 형태의 감소는 STN8 돌연변이 개체의 상태 전이 현상을 감소시킨다는 것을 증명한다.

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