



# Long-term drought resistance in rice (*Oryza sativa* L.) during leaf senescence: a photosynthetic view

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## Abstract

Drought is one of the major abiotic stress that limits crops yield. Here we investigated photosynthetic performance in flag leaves of a hybrid rice (*Oryza sativa* L.) LYPI exposed to prolonged drought stress during leaf senescence. Our results highlighted an architecture remodeling of thylakoid membrane proteins characterizing by amplified PSI-LHCII and PSII dimers supercomplexes and LHCII assemblies played crucial role in drought resistance. Coordination of PSII and LHCII protein phosphorylation, thermal dissipation, and CEF around PSI sustained photo-equilibrium when LEF was suppressed by decreased *Cytb<sub>6</sub>f* complex. CO<sub>2</sub> limitation resulted in degradation of Rubisco content and loss of Rubisco activity, but up-regulated the enzymes responsible for C<sub>4</sub> photosynthesis pathway. It was collectively pointed to that redox poise was a pivotal and ATP/NADPH ratio was probably a linker of light reaction, CO<sub>2</sub> assimilation and other metabolism pathways. In general, our results provided additional insights into the role of thylakoid membrane plasticity in long-term ambient acclimation and complex coordination of drought responses in rice LYPI during the senescence stage.

**Keywords** Rice · Drought stress · Leaf senescence · Thermal dissipation

## Introduction

In oxygenic photosynthetic organisms, the thylakoid membrane system harbors the photosynthetic electron transfer chain that converts solar energy absorbed by pigments into chemical energy, in the form of ATP and reducing equivalents NADPH mainly consumed in CO<sub>2</sub> fixation (Garab 2014). For higher plants, such effective photosynthetic machinery is employed by photosystem I (PSI) and photosystem II (PSII), which act in series under sophisticated photo-equilibrium to maintain appropriated ATP/NADPH ratio. It is well established that the serially cooperating photosystems have lateral heterogeneity in their distribution within the thylakoid membranes, PSII predominantly localize in appressed regions, *i.e.* grana stacks, PSI and ATP synthase are confined to the non-appressed, including grana

ends, grana margins and stromal lamellae, while *Cytb<sub>6</sub>f* complex is found unevenly in both, with small electron carriers, plastoquinone (PQ) and plastocyanin (PC) diffused back and forth (Kirchhoff 2014; Pribil et al. 2014). Both PSI and PSII are multisubunit membrane-protein complexes laterally associated with respective peripheral antenna systems, namely pigments-binding light harvesting complexes (LHCs). Actually, the photosynthetic apparatus is more complicated in nature as a result of hierarchical organizations into various supercomplexes and megacomplexes, along with membrane fluidity, offering the flexibility to allow protein reorganization and redistribution (Kirchhoff 2014; Pribil et al. 2014; Garab 2014). Such plasticity is vital for plants adaptation to ambient changes, maintaining essential photosynthetic rate when illumination or CO<sub>2</sub> is altered and performing effective protection or repair processes to minimize the photodamage induced by excess energy (Suorsa et al. 2015).

Plentiful investigations on structure and dynamics of the thylakoid membrane under various conditions have been carried out to reveal the underlying regulative functions. Most prominently, structural and functional plasticity of LHCII plays important regulatory roles in photosynthetic acclimation through mediating state transition by phosphorylation/

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dephosphorylation (Garab 2014). This process is initiated when the redox poise of the PQ pool is perturbed by unequal excitation of PSII and PSI. Preferential excitation of PSII results in protonation of PQ and facilitates the interaction of PQH<sub>2</sub> and the Q<sub>0</sub> site of Cytb<sub>6</sub>f complex, thereupon activating the STN7 kinase responsible for LHCII phosphorylation, which consequently disassociates from PSII and exhibits higher affinity to PSI (state II) (Goldschmidt-Clermont and Bassi 2015). Conversely, when PQ is deprotonated, STN7 becomes inactive and LHCII is dephosphorylated by the constitutively active TAP38/PPH1 phosphatase, thus recombined with PSII (state I). It is noteworthy that state transition was considered as a short-term response while changes in PSII/PSI stoichiometry were cardinal in long-term acclimation (Chow 1990). However, other evidences pointed out that LHCII trimer served as an intrinsic light harvester for PSI, and state transition was actually redox-controlled dynamics in PSII-LHCII, LHCII pool and PSI-LHCII (Galka et al. 2012; Grieco et al. 2015). Furthermore, the PSII repair cycle, another crucial photoprotective process, also relies on phosphorylation-dependent disassembly of PSII-LHCII supercomplexes. The disassembly leads to a partial destacking of grana and swelling of lumen, which favors PC-mediated electron transport between Cytb<sub>6</sub>f and PSI, mobilization of photodamaged D1 proteins to stroma lamellae and their accessibility to proteases, and of course LHCII migration in state transition (Puthiyaveetil et al. 2014; Giovanardia et al. 2018). Compared with the rapid turnover of PSII, PSI seems to be more vulnerable to photodamage as its recovery is much slower. To address the possible redox pressure at PSI such as acceptor limitation caused by CO<sub>2</sub> dearth, plants have evolved an additional redox-controlled electron transport pathway independent of state transition, cyclic electron flow (CEF) (Takahashi et al. 2013). Two CEF systems in plants have been proposed: the chloroplast NADH dehydrogenase-like (NDH) complex-dependent pathway and the PGR5-PGRL1 protein-dependent pathway. The latter has been indicated the dominating CEF that generates additional ATP and sustains the proton gradient ( $\Delta$ pH) across thylakoid membrane (Shikanai 2016). However, it has been suggested that the NDH-dependent CEF pathway also contributed to proton motive force (*pmf*) for ATP synthesis at low light condition, and was activated by H<sub>2</sub>O<sub>2</sub> (Strand et al. 2015; Yamori et al. 2015). Notably, lumenal acidification ascribed to  $\Delta$ pH is also vital for protective mechanisms in PSII, the non-photochemical quenching (NPQ) which depends on  $\Delta$ pH and is facilitated by the PsbS protein and activation of the xanthophyll cycle (Sylak-Glassman et al. 2014). It has been suggested PsbS protonation promoted disassembly and aggregation of LHCII by interaction with Lhcb1 (Correa-Galvis et al. 2016). Besides, alternative electron transport activities mediated by diverse plastid terminal oxidase also act as a safety valve for excess excitation energy (Laureau

et al. 2013). Nevertheless, intriguingly, PSI with damaged FeS clusters could conduct a self-rescue transformation, where it functions as a powerful non-photochemical energy quencher to alleviate the photoinhibition caused by excess electrons from PSII (Tiwari et al. 2016). Briefly, when linear electron transport is precluded, redox state of PQ initiates relevant protective mechanisms, concomitantly with the suppression of Cytb<sub>6</sub>f complex triggered by lumenal acidification and co-regulation of ATP synthase to modulate the photo-equilibrium, basing on alteration of thylakoid membrane architecture. It has been collectively pointed to a functional integrity of PSI and PSII in spite of their distinct photoprotective manners (Schottler et al. 2015; Yamori et al. 2011).

So far, dynamics of the thylakoid membrane have been mostly investigated under different light conditions and results mainly elucidated short-term acclimation, while much less information has been known in other cases, especially in long-term stress (Kaiser et al. 2015; Ruban and Johnson 2015). The dynamics of thylakoid membranes have been detected under cadmium stress and iron deficiency (Basa et al. 2014), salt stress (Shu et al. 2015) and drought stress (Chen et al. 2016). Among various abiotic and biotic stress, drought is one of the major stress factors that limit crop yield (Cominelli et al. 2013). Drought response network consists of multiple morphological, biochemical and physiological processes. The effects largely depend on duration and severity of drought as well as genotypes and developmental stages of plants (Barnabas et al. 2008). Long-term drought responses of crops have been emphasized on proton and electron transport (Zivcak et al. 2014), protein expression and chloroplast ultrastructure (Vassileva et al. 2012) and enzymatic antioxidant capacity (Huseynova 2012). However, few studies have ascertained the constitutive dynamics of thylakoid membranes to long-term drought stress in rice plants, which is prone to suffer from frequent water deficit. Here, we hypothesized that the re-architecture of thylakoid membrane played a central role in long-term photosynthetic acclimation to drought stress. In view of this, we employed a widely cultured hybrid rice ‘Liangyoupeijiu’ (LYPJ, *Oryza. sativa* L.), well known for its high yield and good grain quality in China and South Asia, and monitored the photosynthetic properties of its flag leaf, the pivotal contributor of grain filling under prolonged drought stress (Yu et al. 1997). Our previous work showed that flag leaves of LYPJ maintains relatively high maximum quantum yield of primary photochemistry (only 0.1–0.2% lower than well-watered group) and stable end electron acceptor reduction in PSI (98–99% of that in control and not-fall, but-rise on 35 days) under drought stress during leaf senescence (Wang et al. 2017a). It was reliable to underline that drought resistance of LYPJ underwent remodeling of photosynthetic apparatus. In the present work, we extended the analyses by

examining the effects of prolonged drought stress on thylakoid membrane component and function to probe elucidation of the antecedent observations. Additionally, given the inevitable CO<sub>2</sub> limitation caused by stomatal closure when encountering water deficit, coordination of photo-carbon balance was also analyzed through assays the activity of photosynthetic enzymes relevant to carbon fixation.

## Materials and methods

### Plant materials and stress treatment

The seeds of rice (*O. sativa* L.) genotype LYP9 were obtained from the Institute of Agricultural Sciences of Jiangsu Nanjing, China. The experiments were conducted during 10 August (first flag leaves expansion) to 26 September (the harvest time) in 2014 and 2015 in Nanjing Normal University, Jiangsu Province, China. According to Wang et al. (2017b), seeds were surface sterilized in 30% H<sub>2</sub>O<sub>2</sub> for 10 min, followed by three thorough washing with sterile distilled water. Subsequently, the seeds were soaked in water for 72 h in dark to induce germination. Seedlings were grown in a climate chamber (16/8 h light [350 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux density (PPFD PAR)]/dark periods, 30/25 °C and 80% relative humidity) until the four-leaf stage. Afterwards, the seedlings were transplanted into 12 plastic pots [two blocks, six pot (four plants each pot) for well-watered condition and six for drought treatment] containing soil from the paddy field. The loam soil was optimally fertilized with available nitrogen, phosphorus and potassium. The total N fertilizer applied was 3.375 t ha<sup>-1</sup>, with an N-P-K ratio of 1: 0.6: 0.6. Drought were initiated on rice with flag leaves fully expanded by withholding water based on appearing drought symptoms such as leaf angle change. Optimal water was supplied to DS plants until the soil was white and cracking, and leaves were wilting and withered. During the experimental period where the whole senescence stage of flag leaves was concluded, samples were collected at about 7-day intervals and stored at – 80 °C until analysis.

### Chlorophyll *a* fluorescence

Measurements of chlorophyll (chl) fluorescence was performed with a pocket fluorometer (*Handy PEA*, Hansatech, UK) between 9:00 AM and 10:00 AM on sunny days in uppermost, fully expanded flag leaves of rice plants as described by Zhang et al. (2015). Leaf clips were dark adapted for 30 min (to obtain open reaction centers) and then irradiated with red light at 3000 μmol m<sup>-2</sup> s<sup>-1</sup> for 1 s. The professional *PEA Plus* and *BioLyzer HP3* software were employed to calculated the fluorescence parameters.

The formulas of fluorescence parameters were referred to those mentioned in Zhang et al. (2015). For the well-watered group and drought stress group, ten flag leaves in different plants for each group were made respectively.

### Two-dimensional blue-native/SDS–polyacrylamide gel electrophoresis

Separation of thylakoid membrane complexes was according to the method of Järvi et al. (2011) with some modifications. Thylakoids were isolated from flag leaves ground in grinding buffer optimized for rice. The chlorophyll concentration of thylakoid fractions was determined as described in Porra et al. (1989). The thylakoid membrane was resuspended into ice-cold storage buffer [25 mM Bis–Tris/HCl (pH 7.0) and 20% (w/v) Glycerol] to a chlorophyll concentration of 1.0 mg ml<sup>-1</sup>. An equal volume of detergent solution was added to a final concentration of 1.5% (w/v) dodecyl-β-D-maltoside (DM) (Sigma, USA). Separation of the thylakoid membrane protein complexes by BN-PAGE was obtained by using 5–12.5% separation gel and 4% stacking gel with electrophoresis system suggested by Järvi et al. (2011). Two-dimensional Blue-native/SDS–polyacrylamide gel electrophoresis (2D BN/SDS-PAGE) was carried out as described by Wittig et al. (2006) with minor modifications. When the BN-PAGE was completed, gel strips of 3 mm wide were cut out from BN-PAGE lanes, and incubated with gentle shaking in solubilizing buffer [66 mM Na<sub>2</sub>CO<sub>3</sub>, 2% (w/v) SDS and 0.67% β-Mercaptoethanol] at 25 °C for 30 min, following with briefly rinsed in distilled water to remove excessive β-mercaptoethanol. Subsequently, the strips were attached to the top of the denaturing gel [15% (w/v) polyacrylamide and 6 M urea] and overlaid in SDS running buffer [25 mM Tris, 190 mM glycine and 0.1% SDS] containing 0.5% (w/v) agarose (Laemmli 1970). Following the electrophoresis, Protein spots were stained with Coomassie Brilliant Blue R-250.

### Image acquisition and data analysis

The stained gels were scanned using the Image scanner III (GE Healthcare). The images were analyzed with Imagemaster™ 2D Platinum software version 6.0 (GE Healthcare). Three gels for each treatment from three independent experiments were used for the analysis. The intensities of spots were quantified based on the ratio of the volume of a single spot to the whole set of spots. Only spots with quantitative changes of at least 1.5-fold in abundance that were reproducible in three replicates were used for mass spectrometry.

### MALDI-TOF/TOF MS analysis and database searching

Differentially expressed protein spots were extracted, processed, and analyzed using an ABI 4800 Proteomics

Analyzer MALDI-TOF/TOF MS (Applied Biosystems, Foster City, CA). Internal mass calibration was performed using trypsin autodigestion products. Database searching and peptide mass fingerprinting (PMF) were performed using the Mascot server (<http://www.matrixscience.com>). Sequences data of differential expressed protein data were searched from NCBI nr (<http://www.ncbi.nlm.nih.gov>) database, with the MOWSE search parameter criteria as follows: trypsin specificity, one missed cleavage site, cysteine carbamidom ethylation, acrylamide modified cysteine, methionine oxidation, similarity of pI, relative molecular mass specified, and minimum sequence coverage of 15%. All identified proteins had a MASCOT score greater than a significance level of  $p < 0.05$ .

### SDS-PAGE and immunoblotting

The isolated thylakoid membrane solution was boiled with loading buffer [125 mM Tris–HCl (pH 6.8), 2 mM EDTA, 30 mM mercaptoethanol, 5% (w/v) SDS, 5% (w/v) sucrose and 0.02% bromophenol blue] for 5 min. For protein immunoblot analysis, isolated thylakoid protein samples with same amount of Chl were first separated by 12.5% SDS-PAGE, then transferred to PVDF membranes and immunoblotted with various antibodies (Agrisera, Sweden).

### CO<sub>2</sub> assimilation enzyme activity assays

The activities of phosphoenolpyruvate carboxylase (PEPC) and NADP-dependent malic enzyme (NADP-ME) were

determined according to Ryšlavá et al. (2003). Total Rubisco activity was cited as described in Wang et al. (2017b).

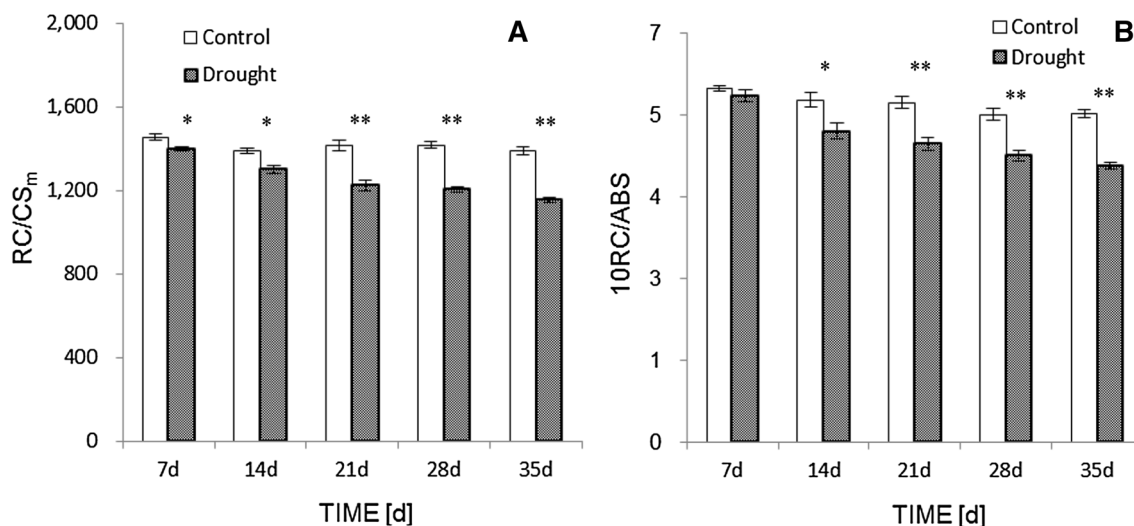
### Statistical analysis

All data were statistically analyzed with SPSS (SPSS Inc., version 18.0, Chicago, USA). The vertical bars represent standard deviations. Differences between treatment means were compared using the LSD at the 0.05 probability level. Single and double asterisks refer to significant difference between the drought and the control at 0.05 and 0.01, respectively.

## Results

### Effects of drought on PSII reaction centers

The amount of PSII reaction centers per excited cross section ( $RC/CS_m$ ) and the total number of active reaction center per absorption ( $10RC/ABS$ ) were measured to dissect the status of PSII reaction centers of rice flag leaves with and without drought treatment. We found that there were significant decreases in both  $RC/CS_m$  (Fig. 1A) and  $10RC/ABS$  (Fig. 1B) with the onset of drought. Both total number of reaction centers and active reaction centers showed extremely significant differences in DS plants and WW plants after 21 days drought treatment. Decrease in active reaction centers was more pronounced in DS plants compared with that of total reaction centers. This phenomenon indicated that less energy was used to drive electron



**Fig. 1** Changes in the amount of PSII reaction centers per excited cross section ( $RC/CS_m$ ) (A) and the total number of active reaction center per absorption ( $10RC/ABS$ ) (B) of flag leaves during senescence stage in high-yield hybrid rice LYPI exposed to drought stress.

Vertical bars represent standard deviations ( $n=10$ ). Single and double asterisks refer to significant difference between the drought and the control at 0.05 and 0.01, respectively



transport in this cultivar LYPJ in this drought condition. Hence, this energy would be dissipated by non-photochemical mechanisms.

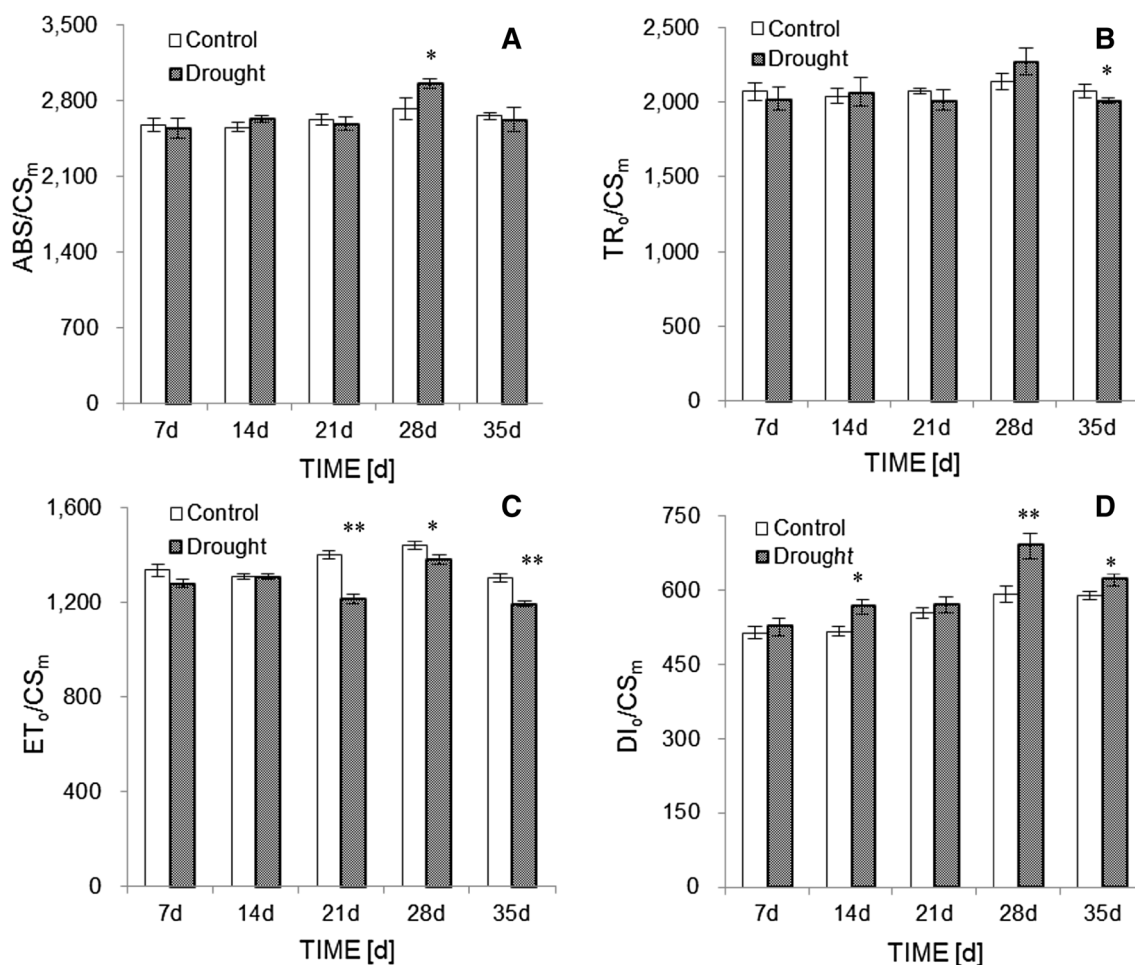
### Effects of drought on PSII photochemical performance

The PSII photochemical performances of rice flag leaves of DS plants and WW plants were characterized by the phenomenological fluxes parameters, including absorption flux of photons per cross section ( $ABS/CS_m$ ), fluxes for trapping per cross section ( $TR_0/CS_m$ ), potential electron transport per cross section ( $ET_0/CS_m$ ) and dissipation per cross section ( $DI_0/CS_m$ ). As evident by Fig. 2, we could notice that, when exposed to drought stress for 28 days, the only significant increase in  $ABS/CS_m$  was concomitant with inhibition of  $ET_0/CS_m$  and increment in  $DI_0/CS_m$ . In general,  $ABS/CS_m$

was relatively little affected by drought but  $ET_0/CS_m$  and  $DI_0/CS_m$  seemed to be down- and up-regulated in DS plants, respectively. The up-regulated dissipation indicators ( $DI_0/CS_m$ ) in DS rice plants in this study suggested that energy dissipation was enhanced in order to protect LYPJ leaves in senescence stage from photo damage.

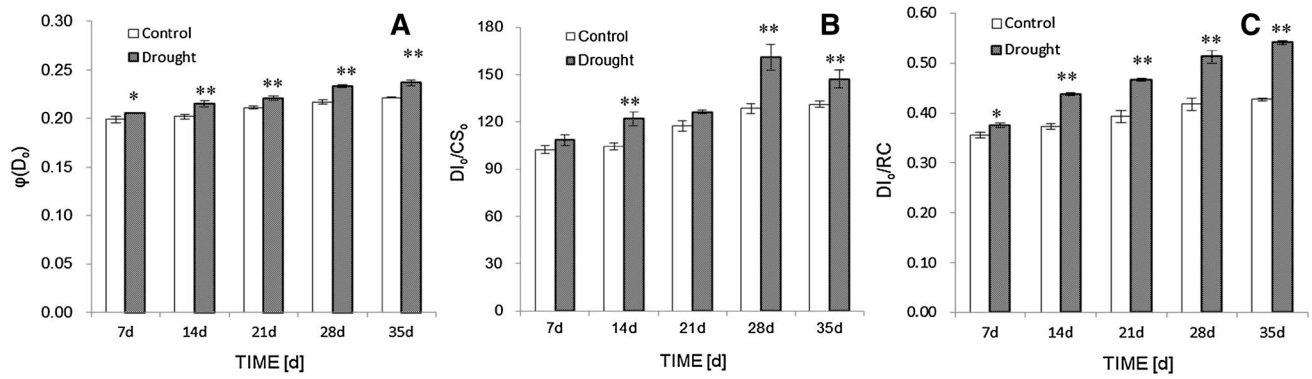
### Drought enhanced the thermal dissipation of PSII

Three thermal dissipation related parameters, thermal dissipation quantum yield ( $\phi D_0$ ), dissipation per cross section ( $DI_0/CS_0$ ) and dissipation energy flux per PSII reaction center ( $DI_0/RC$ ), were presented in Fig. 3. When the treatment time extended,  $DI_0/RC$  of DS plants increased gradually. Likewise,  $\phi D_0$  changed to a higher grade in DS plants and more pronounced when stress accumulated. These changes indicated a lower energy utilization efficiency in DS



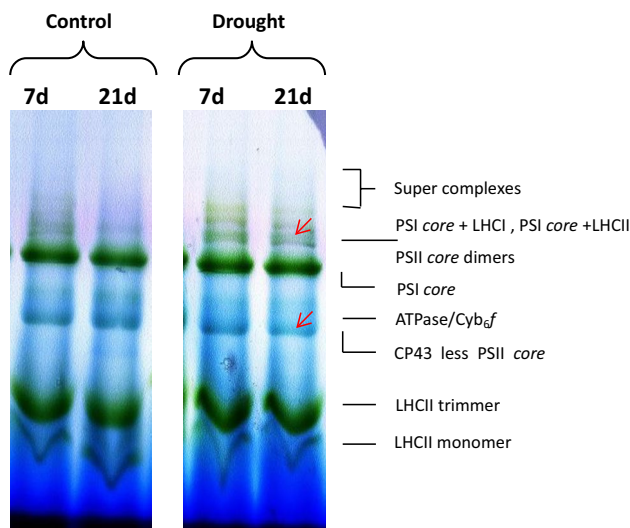
**Fig. 2** Changes in the phenomenological fluxes parameters approximated by  $F_M$ . **A** Absorption flux of photons per cross section ( $ABS/CS_m$ ); **B** fluxes for trapping per cross section ( $TR_0/CS_m$ ); **C** potential electron transport per cross section ( $ET_0/CS_m$ ); **D** dissipation per cross section ( $DI_0/CS_m$ ) of flag leaves during senescence stage in

high-yield hybrid rice LYPJ exposed to drought stress. Vertical bars represent standard deviations ( $n=10$ ). Single and double asterisks refer to significant difference between the drought and the control at 0.05 and 0.01, respectively



**Fig. 3** Changes in related thermal dissipation parameters. **A** Thermal dissipation quantum yield ( $\phi D_0$ ); **B** dissipation per cross section, approximated by  $F_0$  ( $DI_0/CS_0$ ); **C** dissipation energy flux per PSII reaction center ( $DI_0/RC$ ) of flag leaves during senescence stage in

high-yield hybrid rice LYPJ exposed to drought stress. Vertical bars represent standard deviations ( $n=10$ ). Single and double asterisks refer to significant difference between the drought and the control at 0.05 and 0.01, respectively



**Fig. 4** Blue-native PAGE profile of thylakoid membranes isolated from leaves of control and drought plants (each lane was loaded with the same amount of proteins). The analysis was repeated three times with highly similar results

plants, and consistency with the former parameters such as  $DI_0/CS_m$ .

### Effects of drought on thylakoid membrane proteins analyzed by BN/SDS-PAGE

To get an insight into the relationship between thylakoid regulation systems and thylakoid pigment-protein complex dynamics, the patterns of protein complexes from WW and DS rice plants were analyzed. After the first dimension separation by BN-PAGE, five major protein complex bands were observed (Fig. 4), defined as PSI core, ATPase/*Cytb<sub>6</sub>f*, CP43 less PSII core, LHCI trimer and LHCI

monomer. The CP43 less PSII core complex was significantly down-regulated in DS plants. Another primarily change was that drought appeared to decrease the level of LHCI monomer, but with LHCI trimer barely affected. In light of our results, it appeared that PSII core was a preferential target of drought stress and the state of LHCI played an essential role in drought response. Intriguingly, drought induced distinct increment in several bands with high molecular mass. These supercomplexes were further analyzed by mass spectrometry, and subunits of both PSI and PSII were identified, as well as subunits of LHCI.

Information of identified protein spots by BN/SDS-PAGE (21 days-drought-treated rice plants) was shown in Table 1. Of these, six proteins in drought-intensified supercomplexes were found dominated, including CP43 (spots 48 and 50), D1 (spot 46), oxygen-evolving complex protein (OEC) 1 (spots 44 and 45), PsaA (spots 53 and 54), PsaB (spot 55) and chlorophyll a-b binding protein (spot 35). Formation of PSI-LHCI supercomplex and PSII core dimer could be speculated. In PSI core complex, the level of PsaD (spot 0) decreased in DS plants. For PSII core complex, core proteins—D1 (spot 20) and D2 (spots 14 and 15), reduced by drought stress, as well as photosystem II 44 kDa protein (spot 23) and OEE1 (spot 17). Additionally, antenna proteins Lhcb4 (CP29) (spot 41) and Lhcb5 (CP26) (spot 43) were amplified in stress condition while Lhcb1 (CP28) (spot 11) was diminished. The dominant subunit of ATPase—alpha (spot 50) and beta (spot 51), were found to be up-regulated in DS plants. Meanwhile, *Cytb<sub>6</sub>f* (spot 32), which was a critical subunit of *Cytb<sub>6</sub>f* complex, showed remarkable degradation. Last but not least, several non-photosynthetic proteins (spots 31, 36, 37, 38, 40, 42 and 49) located in chloroplast were found differentially expressed under drought stress, which might have regulatory roles.

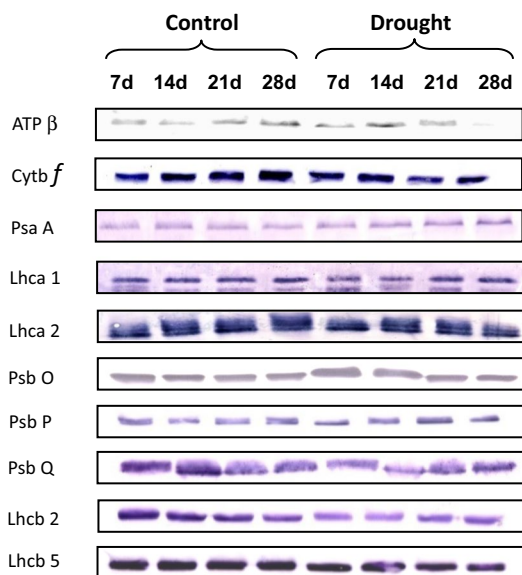
**Table 1** Thylakoid membrane proteins identified from the rice leaf (21 days after drought-treated) by MS

Match ID	Accession no.	Identical protein	Mascot score	Sequence coverage (%)	Number of matched peptides	Mr/pI	Variation
0	gil115477831	Photosystem I reaction center subunit II-like protein, Psa D [ <i>Oryza sativa</i> ]	36	18	2	22.134/9.72	Down
2	gil18425243	Hypothetical protein [ <i>Oryza sativa</i> ]	34	74	2	6.19/9.77	Down
3	gil3789952	Chlorophyll <i>a/b</i> -binding protein presursor [ <i>Oryza sativa</i> ]	70	5	1	27.062/5.59	Down
11	CB21_ORYSJ	Chlorophyll <i>a-b</i> binding protein 1, chloroplastic [ <i>Oryza sativa</i> ]	54	43	7	28.053/5.14	Down
13	USP_ORYSI	UDP-sugar pyrophosphorylase [ <i>Oryza sativa</i> ]	59	18	9	67.819/6.51	Down
14	gil11466770	Photosystem II protein D2 [ <i>Oryza sativa</i> ]	58	2	1	39.776/5.43	Down
15	gil11466770	Photosystem II protein D2 [ <i>Oryza sativa</i> ]	48	2	1	39.776/5.34	Down
17	gil115436780	Oxygen-evolving enhancer protein 1 [ <i>Oryza sativa</i> ]	126	42	11	35.068/6.10	Down
20	gil11466764	Photosystem II protein D1 [ <i>Oryza sativa</i> ]	61	3	1	39.052/5.21	Down
22	ATPA_ORYSA	ATP synthase subunit alpha, chloroplastic [ <i>Oryza sativa</i> ]	62	29	12	55.687/5.95	Up
23	gil11466771	Photosystem II 44 kDa protein [ <i>Oryza sativa</i> ]	75	5	2	52.214/6.71	Down
31	gil125602531	Leucine-rich repeat-containing protein family: zinc-finger homeodomain protein [ <i>Aegilops tauschii</i> ]	52	12	5	105.294/6.17	Born
32	gil11466819	Cytochrome b6 [ <i>Oryza sativa</i> Japonica Group]	123	13	1	24.281/8.89	Down
35	CB23_ORYSI	Chlorophyll <i>a-b</i> binding protein, chloroplastic [ <i>Oryza sativa</i> ]	32	5	1	28.534/5.62	Up
36	gil47847873	Putative CDPK substrate protein 1; CSP1 [ <i>Oryza sativa</i> ]	42	6	3	45.449/5.64	Born
37	gil218197091	BAG family molecular chaperone regulator 8, chloroplastic [ <i>Oryza sativa</i> ]	45	26	3	16.502/5.71	Born
38	gil48926671	Homologous to-Peptide chain release factor 2 [ <i>Arabidopsis thaliana</i> ]	55	22	6	39.453/11.73	Up
40	SAG39_ORYSI	Senescence-specific cysteine protease SAG39 [ <i>Oryza sativa</i> ]	23	11	2	37.445/5.17	Up
41	gil115472753	Type II light-harvesting chlorophyll <i>a/b</i> -binding protein Lhcb4 (CP29) [ <i>Oryza sativa</i> ]	130	5	1	31.387/5.33	Up
42	gil218197348	Hypothetical protein OsI_21184, TPR repeated containing protein family, chaperone response to stress [ <i>Oryza sativa</i> ]	52	11	6	74.262/8.94	Born
43	gil62733870	Chlorophyll <i>a/b</i> -binding protein Lhcb5 (CP26) precursor [ <i>Oryza sativa</i> ]	165	13	2	24.317/5.95	Up
44	gil739292	Oxygen-evolving complex protein 1 [ <i>Oryza sativa</i> ]	93	15	2	26.603/5.13	Up
45	gil739292	Oxygen-evolving complex protein 1 [ <i>Oryza sativa</i> ]	143	15	2	26.603/5.13	Up
46	PSBA_ORYSA	Photosystem II protein D1 [ <i>Oryza sativa</i> ]	40	3	1	39.052/5.21	Up
48	PSBC_ORYSA	Photosystem II CP43 reaction center protein [ <i>Oryza sativa</i> ]	60	18	8	52.214/6.71	Up
49	gil125592049	Serine/threonine protein kinase receptor [ <i>Oryza sativa</i> ]	60	27	7	47.738/6.25	Born
50	PSBC_ORYSA	Photosystem II CP43 reaction center protein [ <i>Oryza sativa</i> ]	56	17	7	52.214/6.71	Up

**Table 1** (continued)

Match ID	Accession no.	Identical protein	Mascot score	Sequence coverage (%)	Number of matched peptides	Mr/pI	Variation
51	ATPA_ORYSA	ATP synthase subunit alpha, chloroplastic [ <i>Oryza sativa</i> ]	35	14	4	55.678/5.95	Up
52	gil50233978	ATP synthase CF1 beta subunit (chloroplast) [ <i>Oryza nivara</i> ]	240	63	29	53.978/5.38	Up
53	gil11466787	Photosystem I Psa A [ <i>Oryza sativa</i> ]	100	2	2	83.56/6.83	Up
54	gil11466787	Photosystem I Psa A [ <i>Oryza sativa</i> ]	70	1	1	83.56/6.83	Up
55	gil11466786	Photosystem I Psa B [ <i>Oryza sativa</i> ]	114	3	2	82.622/6.63	Up

All spots were identified by LC–MS/MS, which was identified by MALDI-TOF



**Fig. 5** Immunoblot with antibodies against photosynthesis-associated protein of thylakoids isolated from flag leaves during senescence stage in high-yield hybrid rice LYPJ exposed to drought stress. Each lane was loaded with same amount of proteins in our experimental conditions

### Effects of drought on thylakoid membrane proteins analyzed by western blot

To identify drought stress responsive changes in thylakoid membrane proteins and testify the results obtained by BN/SDS-PAGE, we performed western blot analysis of subunits of PSI and PSII complexes as well as those of *Cytb<sub>f</sub>* complexes and ATP synthase complexes in DS and WW rice plants (Fig. 5). Both PSI core (PsaA) and antenna subunits (Lhca1-2) pronounced little changes. Antenna subunits of PSII (Lhcb2, and Lhcb5) degraded at different levels. The compositions of OEC complex, PsbO, but not PsbP, PsbQ and PsbR, showed stability to drought stress (Fig. 5). Moreover, *Cytb<sub>f</sub>* declined with stress aggravated.

Likewise, beta subunit of ATPase decreased when stress deteriorated but increment was observed in early (Fig. 5).

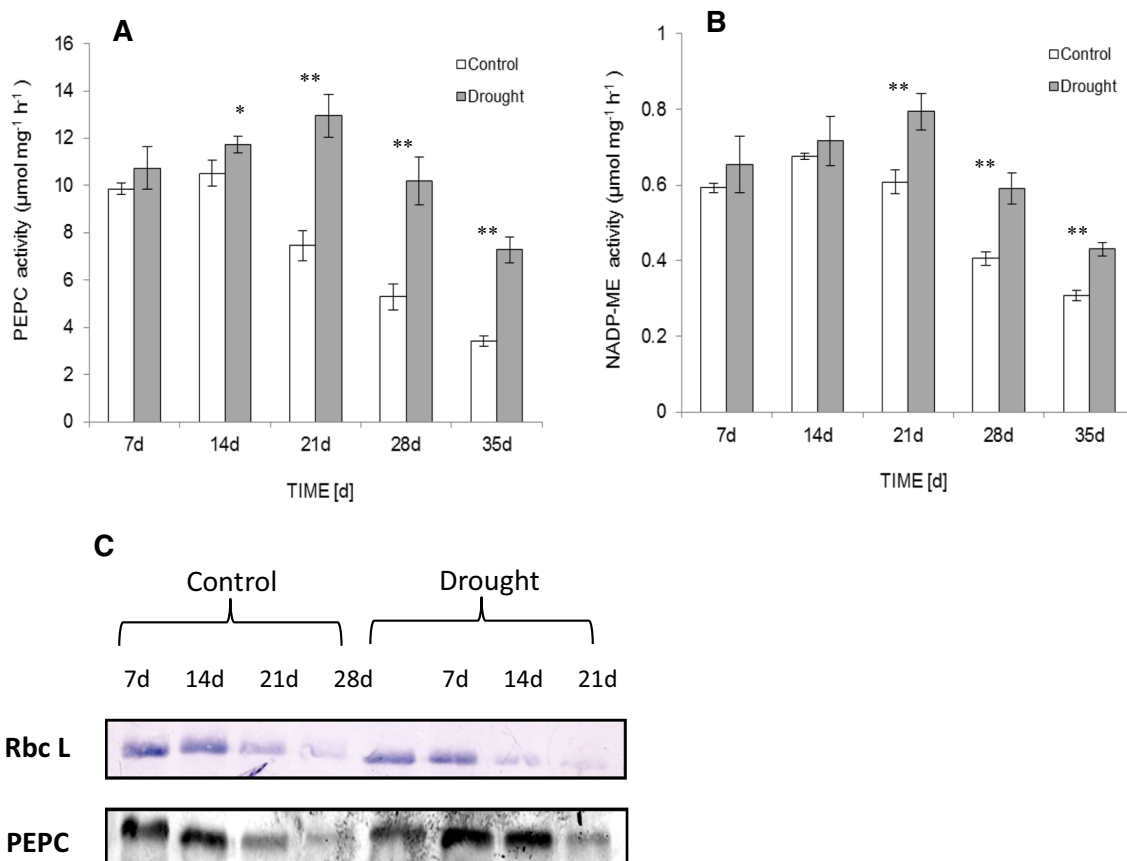
### Effects of drought on photosynthetic enzyme activities

The activities of key enzymes in  $C_3$  pathway represented by Rubisco and  $C_4$  pathway characterized by PEPC and NADP-ME were assessed in WW and DS plants as evident by Fig. 6. Drought stress caused a decrease in the activity of Rubisco, with significant difference arose at the outset in our previous work (Wang et al. 2017b). At translation level, the expression of RcbL was inhibited under drought condition and more obvious after 14 days (Fig. 6C). Conversely, both PEPC and NADP-ME activities increased in DS plants, although significant difference was not found until 21 days (Fig. 6A, B). Moreover, the expression pattern of PEPC was consistent with enzyme activity detection (Fig. 6C).

### Discussion

To improve crop drought tolerance for global food security, we need more profound understandings of strategies adopted by plants to mitigate detrimental effects caused by drought stress (Barnabas et al. 2008; Cominelli et al. 2013). In this study, we attempted to comprehending the mechanism of drought tolerance in the hybrid rice LYPJ, which exhibited impervious photosynthetic efficiency under prolonged drought stress during reproductive stage as previously mentioned, emphasizing on photosynthetic processes. It is interesting to underline that high hierarchical organization of photosynthetic apparatus, PSII core dimer and PSI-LHCII supercomplex were more pronounced in DS plants (Fig. 4). Therefore, it was convincible to propose that the remodeling of thylakoid membrane played a central role in long-term photosynthetic acclimation to drought stress.





**Fig. 6** Activities PEPC (A), NADP-ME (B) and immunoblot with antibodies against large subunit of Rubisco complexes and PEPC subunits (C) in flag leaves during senescence stage in high-yield hybrid rice LYPI exposed to drought stress. Vertical bars represent standard

deviations ( $n=3$ ). Single and double asterisks refer to significant difference between the drought and the control at 0.05 and 0.01, respectively

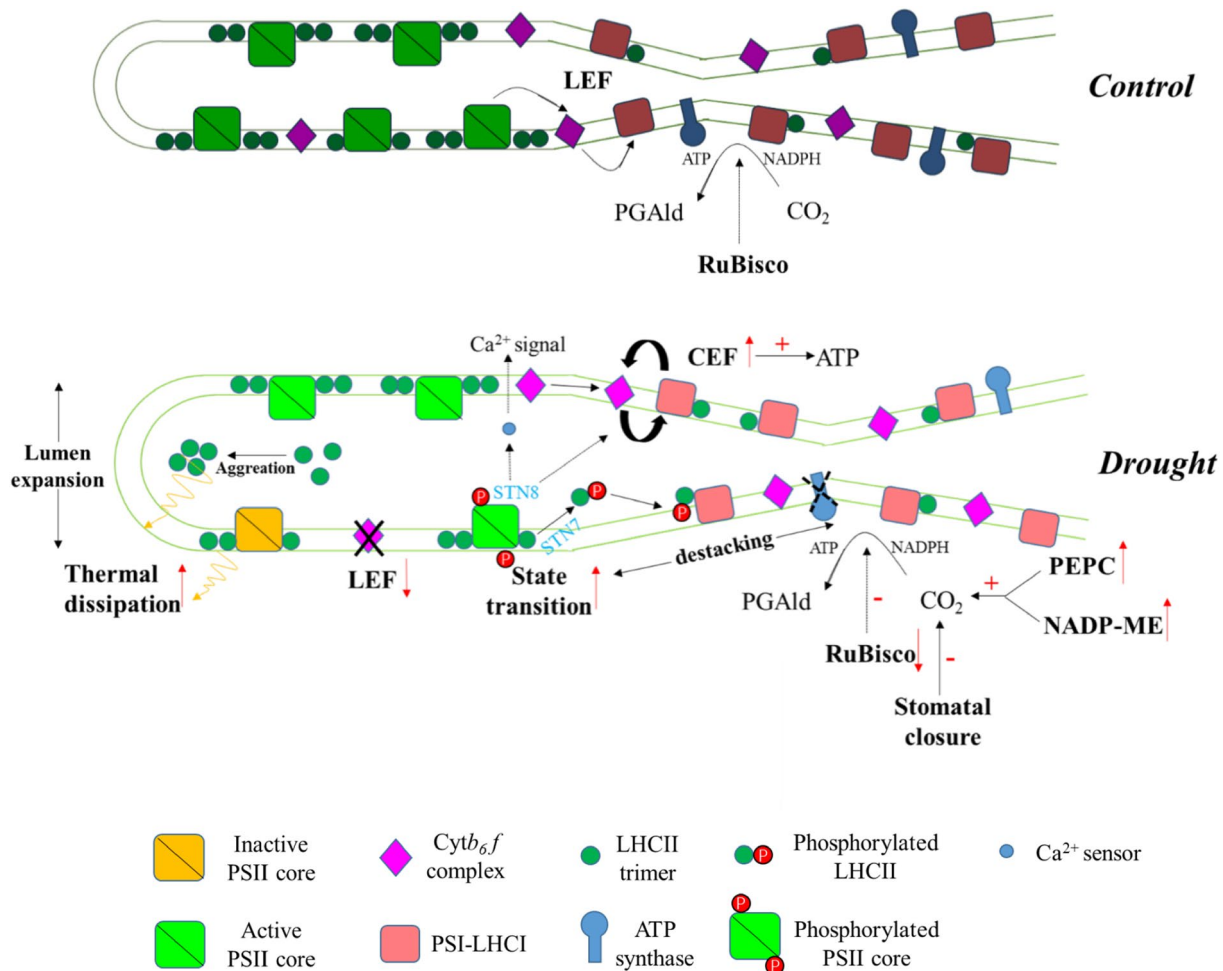
### Photo-equilibrium depended on architecture switch of thylakoid membrane complexes

The efficiency of photosynthesis depends on the coordinated interaction of PSII and PSI in the electron-transport. Plants subjected to the water deficit showed lower values of  $ET_0/CS_m$  characterizing limitation on linear electron flow (LEF) (Fig. 2). This observation could be partially ascribed to deficit of intersystem electron transporter, *Cytb<sub>6</sub>f* complex (Fig. 5, Table 1). Consequently, it would lead to increase of redox poise at PSII acceptor side and PSI donor side, which further gave rise to reactive oxygen species (ROS). To avoid possible photodamage caused by ROS, DS plants underwent serial architecture switch of thylakoid membrane complexes (illustrated by responsive pattern of 21 days drought-treated plants) (Fig. 5, Table 1). First of all, the CP43 less PSII core protein complex was significantly down-regulated, consisting of D1 protein (spot 20) and D2 protein (spots 14 and 15). Conversely, D1 protein in PSII core dimer (spot 46) was higher expressed (Table 1). However, reverse observations were acquired recently in *Arabidopsis thaliana*

exposed to long-term drought stress, where PSII dimer but not PSII monomer decreased with the onset of stress (Chen et al. 2016). This discrepancy might be attributed to the level of D1 phosphorylation which suggested to stabilize PSII dimer, as they reported remarkable reduction of P-D1 in drought-treated plants (Kruse et al. 1997). Therefore, we hypothesized that P-D1 level was sustained or even enhanced in DS plants. Additionally, D1 phosphorylation is also a prerequisite for controlled turnover of the PSII, and thus rapid PSII repaired cycle could be facilitated (Tikkanen et al. 2008). On the other hand, LHCII assemblies altered in DS plants, characterizing by significant decrease in LHCII monomers *i.e.* decrease in LHCII monomer to trimer ratio. Similar results in drought-treated *Arabidopsis thaliana* were reported by Chen et al. (2016), while preferential attack of LHCII trimer was observed in *Beta vulgaris* under cadmium stress and iron deficiency, and decrease in LHCII monomer concomitant with LHCII trimer increase was claimed in salt-treated *Cucumis sativus* (Basa et al. 2014; Shu et al. 2015). This indicated that changes of LHCII assemblies may be stress-specific and/or species-specific.

The possible elucidation of the different performance of monomer and trimer in this work was disassembling of PSII-LHCII supercomplexes suggested by shrinkage and destacking of grana thylakoids (Fig. 7), which partially confirmed by increase of PSII dimers. It was suggested that energy absorbed by monomeric Lhcs can be more easily quenched than trimers (Garab et al. 2002). Intriguingly, although monomeric Lhcb1 showed reduction in DS plants, Lhcb4 and Lhcb5 were significantly amplified (Table 1). Phosphorylation of Lhcb4 by STN7 protein kinase has been indicated an extra dissipative process except PsbS-mediated NPQ in rice (Betterle et al. 2015). Hence, more pronounced monomeric Lhcb4 (probably P-Lhcb4) and Lhcb5 favored the energy

quenching process, in agreement with enhanced thermal dissipation indicated by  $\phi D_0$  (Fig. 3). Meanwhile, decrease in monomeric Lhcb1, we supposed, signified LHCII trimer accumulation although LHCII trimer band showed no difference in DS plants. The elevated PSI-LHCII supercomplex supported the inference (Fig. 5). Both thermal dissipation and formation of PSI-LHCII supercomplex were dependent on phosphorylation of LHCII. P-LHCII, primarily Lhcb1 and Lhcb2 (Crepin and Caffarri 2015; Longoni et al. 2015), concomitantly with PSII core protein phosphorylation, facilitated the dissociation of PSII-LHCII which was an imperative step for PSII repair cycle. More important is the benefit that P-LHCII tend to associate with PSI, then readjusting



**Fig. 7** Scheme of drought resistance on photosynthesis in flag leaf of high-yield hybrid rice LYPJ showing structural and functional readjustments of thylakoid membrane complexes and enzymes. Under drought stress, stomatal closure imposes restriction on available  $\text{CO}_2$ . The co-regulation of *Cytb<sub>6</sub>f* complex and ATP synthase cut down the normal supply of ATP and NADPH. Inactivation of PSII reaction center and disassembly of PSII-LHCII favor the thermal dissipation, resulting in destacking of grana. Phosphorylated LHCII trimers migrate from PSII to PSI, maintaining the cross section homeostasis

of dynamic photo-equilibrium. Enhanced cyclic electron flow (CEF) in margins generated additional ATP and sustained the proton gradient ( $\Delta\text{pH}$ ) characterizing by lumen expansion, facilitating the  $\Delta\text{pH}$ -dependent thermal dissipation and diffusion-dependent electron transport by PQ and PC. STN8 protein kinase phosphorylates PSII core, stabilizing its dimeric formation, and involves in regulation of CEF and  $\text{Ca}^{2+}$  signal transduction. The high expression of PEPC and NADP-ME may compensate the decreased Rubisco for  $\text{CO}_2$  assimilation and benefit other metabolism pathways

the spectral and spatial absorption cross-sections of the two photosystems and ensuring homeostatic regulation of the redox poise of electron transfer chain (Galka et al. 2012).

It should be pointed out that only a fraction of P-LHCII migrated to PSI and most disconnected LHCII probably formed aggregations at energy-dissipative state, which were more efficient in dissipation than monomeric and dimeric complexes (Goldschmidt-Clermont and Bassi 2015; van Oort et al. 2007). Whereas in DS plants Lhcbs decreased to varying degrees (Fig. 5) and PSII reaction centers were impaired (Fig. 1), constraints on energy absorption and trapping could yet be regarded as a protection from photodamage. These results suggested that drought posed limitation on electron transport and the excess excitation energy absorption in PSII could be dissipated as thermal dependent on changes in LHCII assemblies.

Limitation of LEF and CO<sub>2</sub> and increment in antenna size though LHCII binding brought pressure for PSI (Tsabari et al. 2015). As already mentioned, the reduction of end electron acceptor of PSI in DS plants was relatively imperious even though CO<sub>2</sub> assimilation was limited. Hence, it was convincible that CEF operated for protecting PSI from photoinhibition. It has been well-documented that CEF plays a crucial role in regulating photo-equilibrium, as it generates additional ATP and sustains ΔpH (Kohzuma et al. 2009). Although Takahashi et al. (2013) demonstrated that CEF was independent of state transition, yet we found they had cooperation in homeostatic regulations. The observed typical thylakoid structure of state II (Fig. 7) with swollen lumen supported the CEF in grana margins through accelerated the diffusion of PQ (Kirchhoff 2014). Likewise, PSII repair taking place in stroma lamellae was facilitated. Lumen acidification was not only a positive feedback of CEF, but also an imperative of PsbS-mediated NPQ. Nonetheless, we still noticed some minor but interesting alterations in PSI. On the one hand, PsaD protein in PSI core complex was down-regulated while PsaA was steady. As PsaD has been indicated of impact on electron transfer between FeS-clusters in PsaC, the changes might play a regulatory role in CEF. Recently, Tiwari et al. (2016) has reported that damage of FeS-clusters transformed into energy quencher in the absence of ΔpH-dependent regulatory mechanisms in *pgr5* mutant. However, whether proteins of PSI participate in controlling CEF has not been determined. On the other hand, Lhca3 decreased in early stress but increased latter, whereas other LhcAs remained unaffected (Fig. 5A). Here we speculated that Lhca3 was a potential regulator of dynamics between PSI-LHCII and LHCII pool, as it was required for complete state transition (Benson et al. 2015). Additionally, TPR repeated containing protein (spot 42), which protects PSI from oxidative disruption during assembly (Heinzel et al. 2016), showed specific expression in DS plants, along with peptide chain release factor 2 (spot 38) and BAG family

molecular chaperone regulator 8 (spot 37). These auxiliary proteins were probably important for the stabilization of PSI-LHCII supercomplex. Briefly, increased PSI-LHCII complex and CEF around PSI were vital for balance of excitation energy and ΔpH-dependent protective mechanisms in PSII. Furthermore, we found that PsbO was not so sensitive to drought stress as PsbP, PsbQ and PsbR (Fig. 5B).

Afterwards, contradictory results arose in ATP synthase, where protein immunoblotting pointed to reduction in expression level, but BN/SDS-PAGE supported an increase pattern (Fig. 5A, Table 1). Kohzuma et al. (2009) considered that ATP synthase was the key control point of photosynthetic proton circuit, while Yamori et al. (2011) demonstrated that LEF was more limited by Cytb<sub>6</sub>f complex. Most documents supported the view that contents of ATP synthase and Cytb<sub>6</sub>f complex were strictly co-regulated (Schottler et al. 2015). Decline of ATP synthase benefited the build-up of ΔpH utilized by NPQ. From another aspect, sufficient ATP was essential for other protective processes such as photorespiration (Silva et al. 2015). Clearly further studies on expression level and direct assay on activity of ATP synthase are in demand.

In conclusion, these results highlight the importance of dynamics of thylakoid membrane complex in rice plant adaptation to water deficit. The redox-controlled and phosphorylation-dependent remodeling of thylakoid membrane lay the foundation for photo-equilibrium, and sustaining ΔpH seemed to a critical necessity for photoprotective process. NPQ and state transition were concomitant in drought response, incompatible with the argument that state transition functioned under low light condition while NPQ was dominant in high light stress (Tikkanen et al. 2010). In favor of Vallon et al. (1991), our results suggested that state transition was not only a light-acclimation mechanism but also contributed to rerouting of photosynthetic electron flow, accompanied by CEF under drought stress.

### Photo-carbon balance demanded enzymes responsible for C<sub>4</sub> photosynthesis

As already discussed, balanced activity of the two photosystems was warranted by the remodeling of thylakoid membrane complex. In particular, co-regulation of Cytb<sub>6</sub>f complex and ATP synthase could be considered as an automatic adaptation for CO<sub>2</sub> limitation caused by stomatal closure. Here we noticed remarkable decrease of Rubisco activity in DS plants in our previous work (Wang et al. 2017b), in agreement with results observed in drought-stressed wheat emerged from Vassileva et al. (2012). According to Galmes et al. (2013), Rubisco-related parameters were not sensitive to mild-to-moderate drought stress and inhibition of Rubisco activity was ascribed to both decreased Rubisco content and increased tight-binding inhibitors (Parry et al. 1997). Likewise, loss of Rubisco

activity and content were more pronounced after 21 days when stress was severe. Yamane et al. (2012) demonstrated that Rubisco could be excluded from chloroplast and degraded though this pathway in salt-treated rice. We proposed that it was partially responsible for decrease of RbcL (Fig. 6C). By virtue of Rubisco degradation, drought-induced nutrient starvation in DS plants might be alleviated as Rubisco was a N pool due to its high abundance (Warren et al. 2004). Notably, potentiated PEPC and NADP-ME activities in DS plants indicated an alternative pathway to address CO<sub>2</sub> limitation. PEPC and NADP-ME are typical enzymes responsible for C<sub>4</sub> photosynthesis, yet C<sub>3</sub> plants possess all of these genes although expression levels are much lower than those in C<sub>4</sub> species (Hibberd and Quick 2002). Doubnerová and Ryšlavá (2011) have reviewed the functions of these C<sub>4</sub> enzymes in C<sub>3</sub> plants and concluded that these enzymes are more important for plants under stress conditions. Besides compensation for CO<sub>2</sub> assimilation, non-photosynthetic counterparts of these enzymes play important roles in replenishment of Krebs cycle intermediates, facilitating the biosynthesis of amino acids and other compounds and NADPH for the antioxidant system (Doubnerová and Ryšlavá 2011). However, the increase of C<sub>4</sub> enzymes seemed to be latter than loss of Rubisco activity, mechanistic regulation of C<sub>4</sub> pathway in C<sub>3</sub> plants has not been clearly determined. By the way, BAG family molecular chaperone regulator 8 (spot 37), a specific protein induced by drought, is a chaperone regulator for heat shock protein (HSP) 70. Vassileva et al. (2012) suggested an important role of HSP70 in drought resistance. Apart from the hypothesis that HSP70 helped stabilize PSII dimer and PSI-LHCII super-complexes, it was of interest to mention that HSP70 could associate with NADP-ME and modulated its catalytic properties (Lara et al. 2005), and thereby HSP70 was probably a candidate regulator of C<sub>4</sub> photosynthesis in C<sub>3</sub> plants under drought stress. Nevertheless, we failed to know the status of photorespiration which is important in stress acclimation as it also provides intermediates for secondary metabolism, such as phenolic compounds observed in cell wall of DS plants which was beneficial for drought resistance. It could be speculated that photorespiration rate changed in DS plants because of alteration in [CO<sub>2</sub>]/[O<sub>2</sub>] and it participated in the regulation network (Silva et al. 2015). Given the importance of ATP/NADPH ratio in controlling these processes but uncertainty of changes in ATP synthase, further investigations should be designed to detect how the ATP/NADPH budget counted.

## Conclusion

Efficient electron transfer reactions require a balanced excitation energy distribution between PSII and PSI. Our results provide evidence that the interactions between PSII and LHCII protein phosphorylation, thermal dissipation,

and CEF around PSI function in balancing the excitation energy distribution between PSII and PSI though architecture remodeling of thylakoid membrane proteins. Additionally, enhanced C<sub>4</sub> photosynthesis may have dual role that it provides compensation for carbon fixation when both CO<sub>2</sub> and Rubisco activity are limited and favors the antioxidant system though supplying important counterparts. In general, more efforts should be donated to determine how ATP/NADPH influenced the integrated mobilization of photosynthesis, CO<sub>2</sub> assimilation, antioxidant system and other energy metabolism pathways in drought responses.

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**Author Contributions** CGX conceived and designed the experiments. XC performed the experiments. WYW analyzed the data. LH contributed analysis tools. WYW and XC contributed to the writing of the manuscript. All of the authors read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declared that they have no conflict of interests to disclose.

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