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Evidence for the role of cyclic electron flow in photoprotection for oxygen-evolving complex☆

Wei Huang[a,b,*,1], Ying-Jie Yang[b,1], Hong Hu[b], Shi-Bao Zhang[b], Kun-Fang Cao[a]

[a] Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan 666303, China
[b] Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650201, China

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ABSTRACT

Cyclic electron flow (CEF) alleviates PSII photo-inhibition under high light by at least two different mechanisms: one is liked to thermal energy dissipation (qE) and the other one is independent of qE. However, the latter mechanism is unclear. Because the photodamage to PSII primarily occurred at the oxygen-evolving complex (OEC), and the stability of OEC is dependent on proton gradient across thylakoid membrane (ΔpH), we hypothesize that the CEF-dependent generation of ΔpH can alleviate photodamage to OEC. To test this hypothesis, we determined the effects of antimycin A (AA), methyl viologen (MV), chloramphenicol (CM), nigericin (Nig) on PSII activity and the stability of OEC for leaves of a light-demanding tropical tree species *Erythrophleum guineense* by the analysis of OKJIP chlorophyll *a* fluorescence transient. After high light treatment, the stronger decrease in F_v/F_m in the AA-, CM-, MV-, and Nig-treated samples was accompanied with larger photo damage of OEC. The AA-treated samples significantly showed lower CEF activity than the H_2O-treated samples. Although the AA-treated leaves significantly showed stronger PSII photo-inhibition and photo-damage of OEC compared to the H_2O-treated leaves, the value of non-photochemical quenching did not differ between them. Therefore, CEF activity was partly inhibited in the AA-treated samples, and the stronger PSII photo-inhibition in the AA-treated leaves was independent of qE. Taking together, we propose a hypothesis that CEF-dependent generation of ΔpH under high light plays an important role in photoprotection for the OEC activity.

1. Introduction

It has been indicated that cyclic electron flow around photosystem I (CEF) is essential for photoprotection in higher plants on condition of excess light energy (Heber and Walker 1992; Clarke and Johnson 2001; Makino et al., 2002; Munekage et al., 2002, 2004; Johnson, 2005). It has been reported that CEF-dependent generation of a proton gradient across the thylakoid membrane (ΔpH) is necessary for the activation of non-photochemical quenching (NPQ) under high light (Munekage et al., 2002, 2004; Nandha et al., 2007; Takahashi et al., 2009; Joliot and Johnson, 2011). Plants dissipate excess light energy as heat through NPQ to diminish the generation of reactive oxygen species (ROS) (Demmig-Adams 1990; Niyogi et al., 1997, 1998, 2001; Li et al., 2002). CEF mutants (*pgr5*) and NPQ mutants (*npq1* and *npq4*) of *Arabidopsis thaliana* showed similar NPQ values under high light, but *pgr5* mutants displayed stronger PSII photo-damage than *npq1* and *npq4* mutants (Takahashi et al., 2009), indicating that CEF alleviates PSII photodamage at least through two different mechanisms: one is linked to NPQ and the other one is independent of NPQ. However, the latter photo-protective mechanism is unclear.

The photo-damage to PSII primarily occurs at the oxygen-evolving complex (OEC) that is located on the lumenal side of the thylakoid membrane (Hakala et al., 2005; Ohnishi et al., 2005; Oguchi et al., 2011a,b; for review Takahashi and Murata 2008; Takahashi and Badger 2011). Previous study reported that the recovery from the inactivation of the oxygen-evolution complex can be suppressed by calcium-channel blockers, indicating that the

Abbreviations: AA, antimycin A; CEF, cyclic electron flow; CM, chloramphenicol; ΔpH, proton gradient across the thylakoid membrane; ETRI, electron flow through PSI; ETRII, electron flow through PSII; F_v/F_m, the maximum quantum yield of PSII; Nig, nigericin; MV, methyl viologen; NPQ, non-photochemical quenching; OEC, oxygen-evolving complex; PSI, photosystem I; PSII, photosystem II; Y(I), quantum yield of PSI; Y(II), effective quantum yield of PSII; Y(NPQ), the fraction of energy dissipated in form of heat via the regulated NPQ mechanism.

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* Corresponding author at: Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan 666303, China.
E-mail address: huangwei@mail.kib.ac.cn (W. Huang).

[1] These authors contributed equally to this study.

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stability of OEC is dependent on the Ca^{2+} in the lumen of thylakoid membrane (Krieger and Weis 1993). Since acidification of the lumen could drive a Ca^{2+}/H^{+} antiport to sequester Ca^{2+} in the lumen (Ettinger et al., 1999), it is speculated that the generation of ΔpH is necessary for the stabilization of OEC. Under strong light, stomatal closure limits the carbon fixation (Calvin-Benson cycle) and induces the over-accumulation of NADPH, which via feedback depresses linear electron flow (LEF) and triggers CEF. Although water-water cycle can help the generation of ΔpH, the water–water cycle in leaves is not a major alternative electron sink for dissipation of excess excitation energy when CO_2 assimilation is restricted (Driever and Baker, 2011). Thus, the activation of CEF mainly compensates for the deficiency of generation of ΔpH under high light. Once CEF activity was interrupted in *pgr5* mutants, high light induced severer PSII photo-inhibition. Therefore, we hypothesize that CEF alleviates PSII photo-inhibition through protecting OEC against photodamage.

To investigate whether CEF-dependent generation of ΔpH is necessary for the stability of OEC, we examined the effect of antimycin A (AA, to inhibit PGR5-dependent CEF), methyl viologen (MV, to promote electron flow from photosystem I to O_2 and abolish CEF), and chloramphenicol (CM, to inhibit protein synthesis) with combination of the illumination of high light for 1 h on the activities of PSII and OEC for leaves of a tropical tree species *Erythrophleum guineense*. Furthermore, the effect of AA on CEF and NPQ for leaves of *E. guineense* was investigated. Our present study strongly indicates that CEF-dependent generation of ΔpH alleviates photo-damage of OEC under high light, which provides new insight on the protective mechanism of CEF.

2. Materials and methods

2.1. Plant material and growth condition

E. guineense G. Don (Fabaceae), a light-demanding tree species, was used in the present study. Its seedlings exhibit good growth performance in Xishuangbanna Tropical Botanical Garden (21°54′N, 101°46′E). The 3-year-old seedlings of this species cultivated in an open field without water and nutrient stress were used for photosynthetic measurements. The maximum growth irradiance exposing to leaves in summer and winter are 1850 and 1289 μmol photons m^{-2} s^{-1}, respectively. We conducted this study in September 2012 (summer), and the air temperature was about 32/23 °C (day/night temperature).

2.2. Chlorophyll fluorescence and P700 measurements

There are three methods to estimate the activity of OEC: 1) measuring O_2 evolution, 2) measuring electron transport from H_2O to dichlorophenolindophenol (DCIP) via a spectrophotometer, and 3) analyzing fast chlorophyll fluorescence kinetics curve (OJIP). In two original papers about the two-step hypothesis of PSII photoinhibition (Hakala et al., 2005; Ohnishi et al., 2005), the first and second methods has been used to estimate the activity of OEC. The third method has been proposed by Strasser RJ 20 years ago (Guissé et al., 1995) and has been proved to be a reliable method by recent 20-years studies (Srivastava et al., 1997; Strasser 1997; Strasser et al., 2000, 2004; De Ronde et al., 2004; Li et al., 2009). It was reported that the decrease in O_2 evolution was accompanied with the increase in relative fluorescence at K-step (Li et al., 2009). Therefore, in the present study, we examined the OEC activity by measuring chlorophyll a fluorescence transient. Chlorophyll *a* fluorescence transient was determined by a Dual-PAM-100 (Heinz Walz, Effeltrich, Germany) after dark adaptation for 30 min at 25 °C. Each transient obtained from the dark-adapted leaves was analyzed according to the JIP-test by utilizing the following original data (Strasser et al., 2000, 2004): (1) the fluorescence intensity at 30 μs (F_o, when all RCs of PSII are open); (2) the maximum fluorescence intensity (F_m, when all reaction centers of PSII are closed); and (3) the fluorescence intensities at 300 μs (K-step), 2 ms (J-step) and 30 ms (I-step) (Li et al., 2009). The relative variable fluorescence intensity was calculated as: $V_t = (F_t - F_o)/(F_{100\,ms} - F_o)$.

Chlorophyll fluorescence measurements were used to calculate the following parameters: $F_v/F_m = (F_m - F_o)/F_m$, $Y(II) = (F_{m'} - F_s)/F_{m'}$ (Genty et al., 1989), $Y(NPQ) = F_s/F_{m'} - F_s/F_m$ (Hendrickson et al., 2004; Huang et al., 2011), where F_o represents the minimum fluorescence in the dark-adapted state, and F_m and $F_{m'}$ are maximum fluorescence values upon illumination of a pulse (300 ms) of saturating light (10000 μmol photons m^{-2} s^{-1}) in the dark-adapted state and light-adapted state, respectively. F_s is the steady-state fluorescence in light. The ratio F_v/F_m, where $F_v = (F_m - F_o)$ is the variable fluorescence, denotes the maximum quantum yield of PSII (Takahashi et al., 2009); it was measured after 30 min dark adaptation at 25 °C. Y(II) is the effective quantum yield of PSII and Y(NPQ) is the fraction of energy dissipated in form of heat via the regulated NPQ mechanism. In the present study, a 635 nm LED was used as actinic light.

Synchronously with chlorophyll fluorescence measurement, P700 redox state was determined by the saturation pulse method (Klughammer and Schreiber, 1994, 2008). P700 was measured in the dual-wavelength mode (photodetector set to measure 875 nm and 830 nm pulse modulated light) (Klughammer and Schreiber, 2008). Saturation pulses (10000 μmol photons m^{-2} s^{-1}), which were introduced primarily for PAM fluorescence measurement, were applied for assessment of P700 parameters as well. The $P700^{+}$ signals (P) may vary between a minimal (P700 fully reduced) and a maximal level (P700 fully oxidized). The maximum level, which in analogy to F_m is called P_m, was determined with application of a saturation pulse after pre-illumination with far-red light. $P_{m'}$ was also defined in analogy to the fluorescence parameter $F_{m'}$. $P_{m'}$ was determined similarly to P_m, but with background actinic light instead of far-red illumination. The photochemical quantum yield of PSI, Y(I), is defined by the fraction of overall P700 that in a given state is reduced and not limited by the acceptor side. It is calculated as $Y(I) = (P_{m'} - P)/P_m$ (Klughammer and Schreiber, 2008).

2.3. Estimation of photosynthetic electron flow through both PSI and PSII

Electron transport through PSI and PSII were calculated as follows: $ETRI = Y(I) \times PPFD \times p \times dI$, $ETRII = Y(II) \times PPFD \times p \times dII$ (Miyake et al., 2005; Huang et al., 2012a,b), where p is the absorbance (the fraction of the incident light absorbed by leaves), and dI and dII are the fractions of the absorbed light distributed to PSI and PSII, respectively. The p was determined with an USB4000 spectra-suite (Ocean Optics Inc., Dunedin, FL, USA) (Huang et al., 2012a,b). The value of p was calculated as: 1 – reflectance – transmittance and equaled 0.92 ± 0.002 ($n = 5$).

Once CEF was activated, ETRI was larger than ETRII (Miyake et al., 2005; Yamori et al., 2011; Huang et al., 2012a,b; Kono et al., 2014; Huang et al., 2015). If CEF was not activated, the value of ETRI equaled that of ETRII (Kono et al., 2014). It has been indicated that CEF was hardly or slightly activated under low light intensities below 50 μmol photons m^{-2} s^{-1} (Huang et al., 2012a,b; Kono et al., 2014). Therefore, under a low light of 46 μmol photons m^{-2} s^{-1}, we assumed that ETRI = ETRII, thus $Y(I) \times 46 \times 0.92 \times dI = Y(II) \times 46 \times 0.92 \times dII$. From the value of Y(I) (0.54 ± 0.03) and Y(II) (0.72 ± 0.005), dI *and* dII were calculated to be 0.57 and 0.43, respectively. Thus, ETRI and ETRII were estimated using the following equations: $ETRI = Y(I) \times PPFD \times 0.92 \times 0.57$, $ETRII = Y(II) \times PPFD \times 0.92 \times 0.43$. In this study, ETRI—ETRII and

ETRI/ETRII were used to reflect the activation of CEF (Yamori et al., 2011; Gao and Wang, 2012; Huang et al., 2012a,b, 2013; Kono et al., 2014).

During state transition, a reduced redox state of the plastoquinone pool leads to the activation of a protein kinase, which phosphorylates light harvest complex II (LHCII). This phosphorylation event causes the migration of the phosphorylated LHCII to PSI (For review Tikkanen and Aro 2014). Such a change would enhance the light absorption by PSI. If the state transition occurred under high light, the actual CEF would be higher than the value we estimated.

2.4. Photo-inhibitory treatments

To examine the role of CEF in photoprotection in leaves of *E. guineense* illuminated under high light, the effect of methyl viologen (MV, to promote electrons from PSI to O_2 and abolish any CEF) and chloramphenicol (CM, to inhibit protein synthesis) on PSII photodamage was examined (Chow and Hope 2005; Fan et al., 2007, 2008; Takahashi et al., 2009). Mature leaves were vacuum infiltrated with CM (3 mM) in the presence or absence of MV (300 μM) for 3 h in darkness and then were placed on wet tissues and treated at 1000 μmol photons m^{-2} s^{-1} and 25 °C for 1 h. Given that ROS inhibit the repair of PSII activity in *Synechocystis* (Nishiyama et al., 2001, 2004, 2005, 2006, 2011) and *Arabidopsis* (Takahashi et al., 2009), the difference in PSII photoinhibition between the CM and CM + MV-treated samples is mainly linked to the abolishment of CEF-dependent generation of ΔpH. A previous study indicated that the effect of MV on OJIP chlorophyll *a* fluorescence after dark-adaptation was mainly reducing the fluorescence intensity at I-P step, but hardly affecting the fluorescence intensity and relative variable fluorescence at O-K-J step (Schansker et al., 2005). Furthermore, treatment with MV in darkness did not affect the value of F_v/F_m (Schansker et al., 2005).

To examine the role of ΔpH across thylakoid membrane on the stability of OEC, mature leaves were vacuum infiltrated with nigericin (Nig, 100 μM) and exposed to a light at 1000 μmol photons m^{-2} s^{-1} at 25 °C for 1 h. It is well documented that there are at least two distinct pathways of CEF, PGR5-dependent and NDH-dependent pathways. To examine whether PGR5-dependent CEF plays a significant role in photoprotection for OEC, the effect of antimycin A (AA, to specifically inhibit PGR5-dependent CEF, Munekage et al., 2002; Shikanai, 2007) on Y(I), Y(II), Y(NPQ) and OKJIP chlorophyll *a* fluorescence transient was examined. Mature leaves were vacuum infiltrated with H_2O or AA (10 μM) and then were performed for light curve measurements following 20 min light adaption. After light curve measurements, they were exposed to a light at 1000 μmol photons m^{-2} s^{-1} at 25 °C for 1 h. After photoinhibitory treatment, the OKJIP chlorophyll *a* fluorescence transient was examined. During the photo-inhibitory treatments, combination of LED blue and red light (650 nm/455 nm) was used.

2.5. Statistical analysis

The results were displayed as mean values of at least four independent experiments. One-Way ANOVA test was used at $\alpha = 0.05$ significance level to determine whether significant differences existed between different treatments.

3. Results

The role of ΔpH across thylakoid membrane in PSII and the OEC activities was examined by the measurement of chlorophyll *a* fluorescence transient in detached leaves treated with H_2O, AA, CM, MV and Nig. After exposure to the light of 1000 μmol photons m^{-2} s^{-1} at 25 °C for 1 h, the maximum quantum yield of PSII, as judged by F_v/F_m, decreased by 11% in H_2O-treated samples (Fig. 1). In the AA-treated samples, F_v/F_m decreased by 30% after the high light treatment (Fig. 1), indicating that inhibition of PGR5-dependent CEF accelerated PSII net photo-inhibition in leaves of *E. guineense*. In the CM-treated samples, F_v/F_m decreased by 32% after 1 h exposure to the high light (Fig. 1), indicating the repair cycle of PSII activity under high light is an important mechanism for alleviating PSII net photo-inhibition in leaves of *E. guineense*. In the Nig-treated samples, F_v/F_m decreased by 46% after 1 h exposure to the high light (Fig. 1). If the repair of photo-damaged PSII was completely inhibited in the Nig-treated samples, the lower F_v/F_m in the Nig-treated samples was caused by higher rate of PSII photo-damage. If the repair of photo-damaged PSII was partly inhibited in the Nig-treated samples, the lower F_v/F_m in the Nig-treated samples was caused by much higher rate of PSII photo-damage. Therefore, whatever happened, the Nig-treated samples showed significantly stronger PSII net photo-inhibition than the CM-treated samples, indicating that the interruption of generation of ΔpH accelerated the rate of PSII photo-damage. In the CM + MV-treated samples, F_v/F_m decreased by 60% after the above high light treatment (Fig. 1). The significant difference in F_v/F_m between the CM-treated and CM + MV-treated samples indicated that CEF-dependent generation of ΔpH across thylakoid membrane depressed the rate of PSII photo-damage in leaves of *E. guineense*.

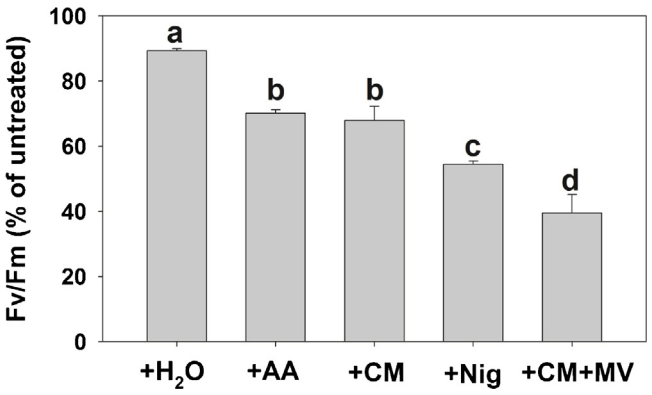

Fig. 1. The effects of methyl viologen (MV), chloramphenicol (CM), nigericin (Nig) and antimycin A (AA) on the decrease in F_v/F_m induced by high light treatment in leaves of *Erythrophleum guineense*. After treated with chemical reagents as described in Section 2, leaf samples placed on wet tissues were illuminated under a high light of 1000 μmol m^{-2} s^{-1} at 25 °C for 1 h. All values were expressed relative to the controls without any treatment. The mean ± SE was calculated from at least four independent plants. Different letters represent significant differences of F_v/F_m between different treatments ($P < 0.05$, One-Way ANOVA).

After exposure to the high light of 1000 μmol photons m^{-2} s^{-1} at 25 °C for 1 h, the relative fluorescence intensity at the K-step (300 μs) significantly increased in the leaves treated with CM, CM + MV, Nig and AA compared to the H_2O-treated samples (Fig. 2). Since the K-step correlates with the damage to the donor side of PSII (Srivastava et al., 1997; Strasser, 1997; Strasser et al., 2000, 2004; De Ronde et al., 2004; Li et al., 2009), the increase in the relative fluorescence at the K-step implies that the donor side of PSII was damaged pronouncedly in the CM-, CM + MV-, Nig- and AA-treated samples than the H_2O-treated samples. Furthermore, the Nig- and CM + MV-treated samples had higher relative fluorescence intensity at the K-step than the CM-treated samples (Fig. 2), suggesting that interruption of generation of ΔpH aggravated photo-damage to the donor side of PSII. As photo-damage to the OEC is responsible for the damage to the donor side of PSII, the increase in the relative fluorescence at the K-step suggested photo-damage of the OEC (Strasser et al., 2000, 2004; De Ronde et al., 2004). Pooling the data obtained after exposure at a high light of 1000 μmol photons m^{-2} s^{-1} and 25 °C for 1 h in leaves treated with H_2O, CM, CM + MV,

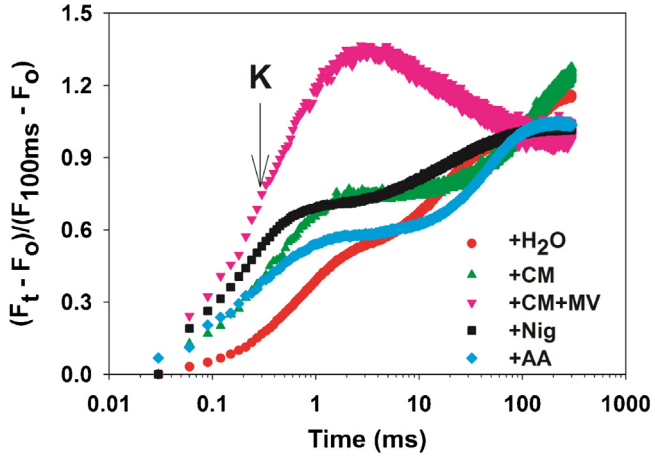

Fig. 2. The effects of methyl viologen (MV), chloramphenicol (CM), nigericin (Nig) and antimycin A (AA) on relative fluorescence intensity (V_t) in leaves of *Erythrophleum guineense*. $V_t = (F_t - F_o)/(F_{100\,ms} - F_o)$. After treated with chemical reagents as described in Section 2, leaf samples placed on wet tissues were illuminated under a high light of 1000 μmol m^{-2} s^{-1} at 25 °C for 1 h. The mean value was calculated from at least four independent plants. The fluorescence intensity at O-step the K-step (300 μs) were significantly higher in the Nig-treated and CM + MV-treated samples than the samples treated with H_2O and CM ($P < 0.05$, One-Way ANOVA).

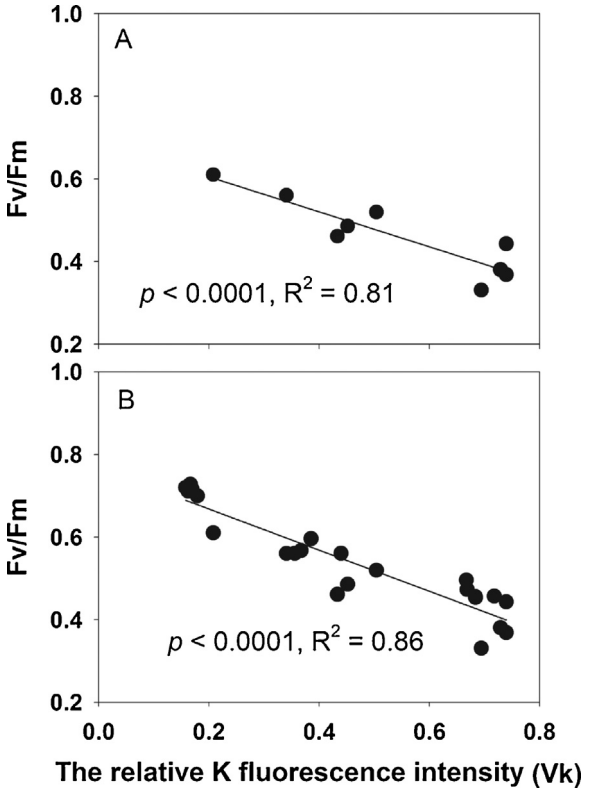

Fig. 3. Change in F_v/F_m as a function of the relative K fluorescence intensity (V_k) after exposure at a high light of 1000 μmol m^{-2} s^{-1} and 25 °C for 1 h in leaves of *Erythrophleum guineense*, which were treated by water, chloramphenicol, methyl viologen, nigericin and antimycin A as described in Section 2. A, treatments with CM and CM + MV; B, treatments with H_2O, CM, Nig, AA, and CM + MV.

Nig and AA, F_v/F_m was strongly and negatively correlated with the relative fluorescence intensity at the K-step (300 μs) (Fig. 3), suggesting the significant correlation between photo-damage of OEC and PSII photo-inhibition.

To examine whether the acceleration of PSII photo-inhibition caused by AA is attributable to depression of NPQ, the effect of AA

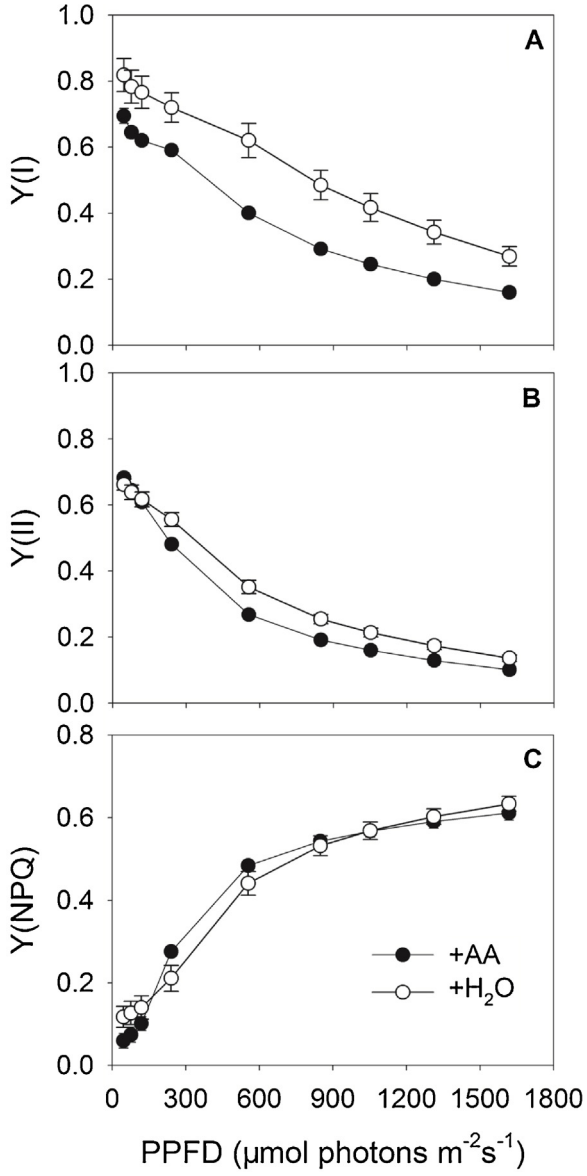

Fig. 4. The effect of antimycin A (AA) on Y(I), Y(II), and Y(NPQ) in leaves of *Erythrophleum guineense* measured at 25 °C. The mean ± SE was calculated from five independent plants. Y(I), quantum yield of PSI photochemistry; Y(II), effective quantum yield of PSII photochemistry; Y(NPQ), fraction of energy dissipated in form of heat via the regulated NPQ mechanism.

on Y(I), Y(II) and Y(NPQ) was measured. Light response curves from the detached leaves without the chemical treatments indicated that with the increase in light intensity, Y(I) and Y(II) gradually decreased as expectedly (Fig. 4A, B). However, values for Y(I) were significantly and largely higher in the H_2O-treated leaves than the AA-treated leaves, irrespective of light intensity (Fig. 4A). Under light intensities above 500 μmol photons m^{-2} s^{-1}, values for Y(II) in the H_2O-treated leaves were significantly higher than that in the AA-treated leaves, but the differences were small (Fig. 4B). With increasing light intensity, Y(NPQ) gradually increased to harmlessly dissipate excess light energy (Fig. 4C). Unlike values for Y(I) and Y(II), light response change in Y(NPQ) was not different between the AA- and H_2O-treated leaves (Fig. 4C). It has been indicated that the activation of thermal energy dissipation is dependent on PGR5-CEF pathway. The pgr5 plants showed much lower values of NPQ and Y(NPQ) than wild type of *A. thaliana* (Munekage et al., 2002, 2004; Takahashi et al., 2009; Kono et al., 2014). The high NPQ val-

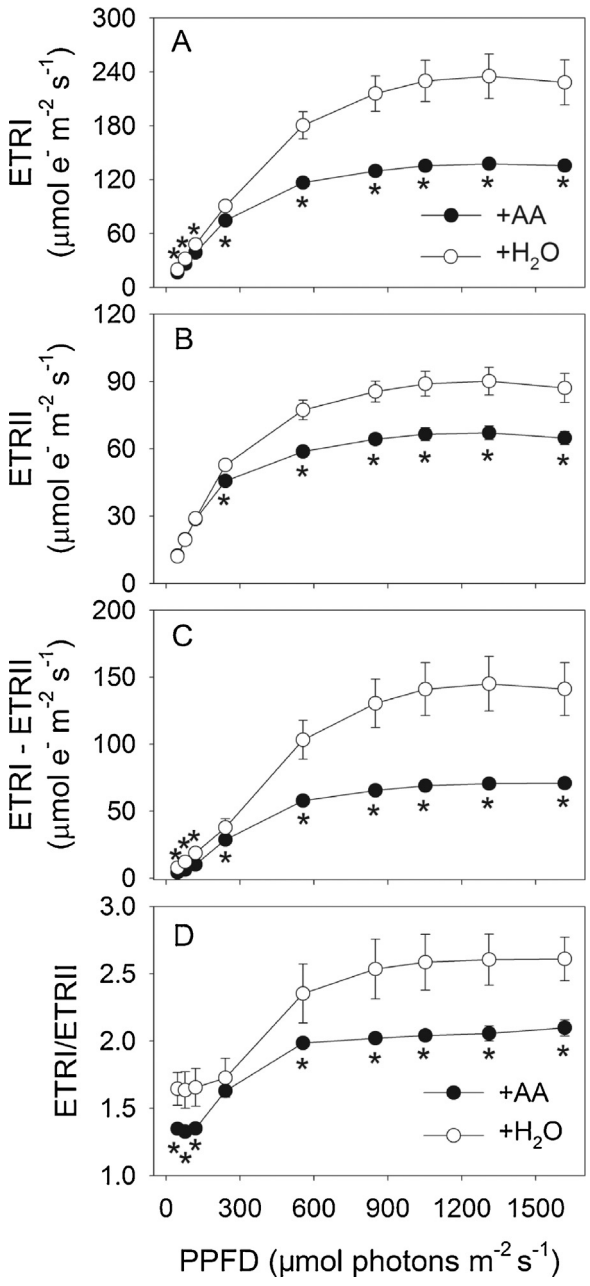

Fig. 5. The effect of antimycin A (AA) on ETR, ETRII, ETRI—ETRII, and ETRI/ETRII in leaves of *Erythrophleum guineense* measured at 25 °C. The mean ± SE was calculated from five independent plants.

ues under high light indicated that the PGR5-CEF was just partly inhibited in the AA-treated samples.

The effect of AA on CEF activation in the studied species *E. guineense* was examined by estimation of ETRI and ETRII. Under low light intensities below 240 μmol photons m^{-2} s^{-1}, values for ETRI and ETRII slightly decreased in the AA-treated leaves compared to the H_2O-treated leaves (Fig. 5A, B). When exposed to light intensities above 240 μmol photons m^{-2} s^{-1}, values for ETRI and ETRII were significantly depressed in the AA-treated leaves (Fig. 5A, B). The values of ETRI—ETRII and ETRI/ETRII were significantly higher under high light compared to low light in both the AA- and H_2O-treated leaves, suggesting the activation of CEF under high light. Interestingly, values for ETRI—ETRII and ETRI/ETRII significantly decreased in the AA-treated leaves (Fig. 5A, B). Because AA is a specific inhibitor of PGR5-CEF pathway, this result indicated that the PGR5-dependent CEF was significantly inhibited in the AA-treated leaves. However, there was no significant difference of Y(NPQ) between the AA-treated and H_2O-treated samples (Fig. 4C), suggesting that the PGR5-dependent CEF activity was partly inhibited in the AA-treated leaves. The remaining CEF-dependent CEF activity maintained the activation of NPQ.

4. Discussion

4.1. CEF alleviates photo-damage of OEC under high light

Although Takahashi et al. (2009) suggested that the PGR5-CEF pathway mainly alleviated PSII photo-inhibition through NPQ-independent mechanism, the underlying mechanism is unclear. We found that antimycin A partly inhibited CEF activity in the studied species *E. guineense*, which did not suppress the activation of NPQ, but accelerated net photo-damage to PSII and OEC. Furthermore, photo-damage of OEC activity was correlated with PSII photo-inhibition. These results indicated that CEF-dependent generation of ΔpH protected OEC from photo-damage against high light stress, and then declined the rate of PSII photo-damage.

Antimycin A specifically inhibits PGR5-dependent CEF (Munekage et al., 2002, 2004; Shikanai 2007). The difference of ETRI-ETRII between AA- and H2O-treated leaves also include Mehler reaction, NDH, P700 charge recombination, and/or electron leakage via PTOX. Generally, the decline in ETRI-ETRII in the AA-treated leaves was mainly caused by the partly inhibition of PGR5-dependent CEF. The generation of ΔpH is determined by LEF and CEF. Under conditions of high rates of CO_2 fixation, in addition to CEF-dependent qE activation, CEF-dependent generation of ΔpH also produce supplementary ATP to meet ATP/NADPH requirements for the Calvin cycle and photorespiration (Yamori et al., 2011; Walker et al., 2014). The same value of CEF can be accompanied with different values of NPQ or Y(NPQ) (Miyake et al., 2005; Huang et al., 2015). When CEF was partly limited, the rate of CO_2 assimilation under high light could be restricted because of lacking ATP. Subsequently, the LEF would rapidly become limiting by the lack of $NADP^+$, decreasing rates of proton translocation and ATP regeneration. As a result, in the AA-treated samples, the contribution of CEF to the synthesis of ATP decreased compared with H_2O-treated samples, and the contribution to the formation of a qE would increase. Furthermore, the depletion of NADP+ can accelerate electron transfer from PSII to O_2 via PSI (water–water cycle), generating ΔpH for qE activation and ATP synthesis. As a result, although the inhibition of CEF by AA decreased the ΔpH, the decreased ΔpH might be partly compensated by the activation of water-water cycle. As a result, the AA-treated samples showed similar Y(NPQ) values compared with the H2O-treated samples.

The higher photodamage of OEC in the AA-treated samples than the H_2O-treated samples indicated the stability of OEC under high light was correlated with CEF-dependent generation of ΔpH. This conclusion was supported by the treatments with methyl viologen, chloramphenicol and nigericin. Methyl viologen promotes electron flow from PSI to O_2, it induces the over-generation of ROS (inhibit the repair of photo-damaged PSII) and abolishes any CEF. The effect of MV on PSII photo-inhibition really remains controversies. Takahashi et al. (2009) reported that MV-induced generation of ROS inhibited the repair of photo-damaged PSII but did not accelerate photo-damage to PSII in *A. thaliana*. However, Krieger-Liszkay et al. reported that superoxide anion radicals generated by MV damage PSII in tobacco. Recently, lots of studies have indicated that the action of ROS in photo-inhibition was to inhibit the recovery rather than accelerate photo-damage. The extent of

PSII photo-inhibition in the CM + MV-treated samples could be regarded as the sum of that in the AA- and CM-treated samples. If the MV-induced ROS cause significantly photo-damage to PSII, PSII photo-inhibition in the CM + MV-treated samples should be higher than the observed result. Chloramphenicol inhibits the repair of photo-damaged PSII through inhibition of D1 protein synthesis. The difference in photo-damage of OEC between the CM- and CM + MV-treated leaves suggests the role of CEF in protecting OEC against photo-damage under high light. Nigericin (Nig) abolishes the generation of ΔpH across thylakoid membrane, which suppressed the activation of NPQ (Takahashi et al., 2009). The difference in photo-damage of OEC between the CM and Nig-treated leaves suggests the role of ΔpH in protecting OEC against photo-damage under high light.

The PSII photo-damage primarily occurs at OEC, which is located the luminal side of the thylakoid membrane (Hakala et al., 2005; Ohnishi et al., 2005). Previous study reported that the recovery from the inactivation of oxygen-evolution can be suppressed by calcium-channel blockers, indicating that the stability of OEC is dependent on the Ca^{2+} in the lumen of thylakoid membrane (Krieger and Weis 1993). Since acidification of the lumen could drive a Ca^{2+}/H^{+} antiport to sequester Ca^{2+} in the lumen, up to about 4 mM in the lumen from an external concentration of 15 μM (Ettinger et al., 1999), it is speculated that the generation of ΔpH across thylakoid membrane is necessary for the stabilization of OEC under condition of excess light. Once the generation of ΔpH across thylakoid membrane was impaired, photo-damage of OEC was accelerated. For example, The CM + MV-treated leaves showed stronger photo-damage of OEC than the CM-treated leaves. The Nig-treated samples showed higher photo-damage of OEC than the CM-treated samples. Although MV and Nig aggravated the production of ROS, the photo-damage of OEC is not cause by ROS (Ohnishi et al., 2005; Oguchi et al., 2011a,b). Therefore, the higher photo-damage of OEC in the MV- and Nig-treated leaves was probably due to impairment of generation of ΔpH across thylakoid membrane.

Under high light, limitation of CO_2 assimilation induces the increase in NAD(P)H/NAD(P)$^{+}$ ratio. Under such condition, the LEF-dependent generation of ΔpH is mainly responsible for ATP synthesis. Therefore, plants must have other flexible pathway to drive the Ca^{2+}/H^{+} anti-port. On condition of excess light energy, the increase in NAD(P)H/NAD(P)$^{+}$ ratio activated CEF. The studied species *E. guineense* has NDH-dependent CEF, as indicated by a transient post-illumination increase in chlorophyll fluorescence (data not shown). It has been indicated that NDH-dependent CEF plays an important role in ATP synthesis under low light (Yamori et al., 2011). Comparing with the *pgr5* plants of *A. thaliana* (Kono et al., 2014), the light response change in Y(NPQ) indicated that *E. guineense* has PGR5-dependent CEF activity. The PGR5-dependent CEF has been regarded as the main CEF pathway to generate ΔpH, which is necessary for the normal activation of NPQ and photo-protection (Yamori et al., 2011). Since CEF was highly activated under high light in the studied plant *E. guineense*, the CEF-dependent generation of ΔpH compensates for the deficiency of LEF-dependent generation of ΔpH, which could increase the concentration of Ca^{2+} in the lumen and then alleviate photo-damage to OEC. Therefore, an important function of CEF was to sequester Ca^{2+} in the lumen and then increase the stabilization of OEC. For plants grown in an open field with high sunlight, CEF is essential for the stabilization of the OEC activity and then prevents severe photo-damage of PSII.

It is also worth noting that AA may affect respiration process, followed by lower ATP production, which consequently slow down the repair of photo-damaged PSII because PSII repair needs ATP energy. As a result, the large difference of photo-inhibition of the OEC and PSII between AA- and water- treated leaves might be partly caused by inhibition of respiration.

4.2. Correlation between photo-damage of OEC and PSII reaction centers

The two-step scheme of PSII photo-damage proposed that the photo-damage of OEC may be a limiting step for PSII photo-damage (Hakala et al., 2005; Ohnishi et al., 2005). However, this scheme remains controversy. In our present study, F_v/F_m was strongly and negatively correlated with the relative fluorescence intensity at the K-step (Fig. 3), indicating the linear correlation between photo-damage of OEC and PSII photo-inhibition. Although the H_2O and AA-treated samples showed the same level of Y(NPQ) (Fig. 1D), after exposure to a high light of 1000 μmol photons m^{-2} s^{-1} at 25 °C for 1 h, F_v/F_m decreased by 30% in the AA-treated leaves and 11% in the H_2O-treated leaves, respectively. Furthermore, the AA-treated leaves displayed stronger photo-damage of the OEC than the H_2O-treated leaves. These results indicated that the higher high-light-induced PSII photo-damage in the AA-treated leaves was not caused by ROS but by photo-damage of the OEC. Based on our results, we speculate that the interruption of generation of ΔpH in the AA-treated leaves primarily induces photo-damage to OEC. Once the OEC was damaged, the electron donation from the OEC to P680 was depressed, which aggravated damage to PSII reaction centers upon light absorbed by photosynthetic apparatus. Previous studies reported that action spectra of photo-damage to PSII showed a strong peak at UV wavelengths toward blue light and no significant peak in red light (Hakala et al., 2005; Takahashi et al., 2010). A recent study indicates that visible light damages OEC prior to photo-damage to the PSII reaction center (Zavafer et al., 2015), which further supports the hypothesis of our present study. Under conditions in which absorbed light is in excess of the requirements for photosynthesis, the increased production of ROS inhibits the repair of photo-damaged PSII. To avoid severe PSII photo-inhibition, CEF-dependent generation of ΔpH favors the stabilization of OEC and then decreases the rate of PSII photo-damage.

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