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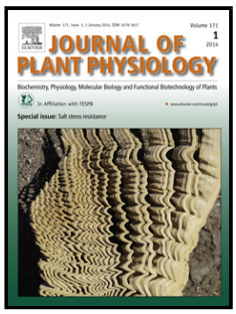

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Specific roles of cyclic electron flow around photosystem I in photosynthetic regulation in immature and mature leaves

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Abstract

Cyclic electron flow (CEF) around photosystem I (PSI) is essential for photosynthesis in mature leaves. However, the physiological roles of CEF in immature leaves are little known. Here, we measured the PSI and PSII activities, light response changes in PSI and PSII energy quenching for immature and mature leaves of *Erythrophleum guineense* grown under full sunlight. Comparing with the maximum quantum yield of PSII (F_v/F_m), the immature leaves had much lower values of the maximum photo-oxidizable P700 (P_m) than the mature leaves, suggesting the unsynchronized development of PSI and PSII activities. Furthermore, the immature leaves displayed significantly lower capacities for the photosynthetic electron flow through PSII (ETRII) and CEF. However, when exposed to high light, the immature leaves displayed higher levels of non-photochemical quenching (NPQ) and P700 oxidation ration [Y(ND)] than mature leaves. Under high light, the similar NPQ values were accompanied with much lower CEF activity in the immature leaves. These results suggest that, in immature leaves, CEF primarily contributes to photoprotection for PSI and PSII via acidification of thylakoid lumen. By comparison, in mature leaves, a large fraction of CEF-dependent generation of ΔpH contributes to ATP synthesis and a relative small proportion favors photoprotection via lumen acidification. These findings highlight the specific roles of CEF in photosynthetic regulation in immature and mature leaves.

Keywords: cyclic electron flow, lumen acidification, non-photochemical quenching, ATP synthesis, photoprotection, photosynthetic control

Introduction

Plants capture light energy to drive photosynthetic electron flow through the thylakoid membranes in chloroplasts. Absorbed light energy makes the oxygen-evolving complex to split water to H^+ and O_2. Electrons released from water in photosystem II (PSII) are transferred through the cytochrome (Cyt) b_6/f complex and photosystem I (PSI) and ultimately to $NADP^+$, resulting in formation of NADPH. Meanwhile, a proton gradient across the thylakoid membrane (ΔpH) was generated through the Cyt b_6/f complex. These protons enable ATP synthesis via the ATP synthase complex. This pathway is usually called as linear electron flow (LEF). The LEF-dependent generation of ATP and NADPH is primarily utilized in the primary metabolism including the Calvin cycle, photorespiration and nitrogen assimilation. In addition, cyclic electron flow (CEF) is another important alternative electron flow and is essential for photosynthesis (Munekage et al. 2002, 2004; Takahashi et al. 2009; Miyake 2010; Suorsa et al. 2012). During CEF, electrons from either NADPH or ferredoxin are cycled around PSI into the plastoquinone pool, which is coupled to the generation of ΔpH but does not reducing $NADP^+$ (Johnson 2011). The CEF-dependent generation of ΔpH has two main roles: one is linked to ATP synthesis and balances ATP/NADPH ratio (Avenson et al. 2005; Yamori et al. 2011; Nishikawa et al. 2012; Walker et al. 2014; Huang et al. 2015), the other is dependent on lumen acidification and favors photoprotection for PSI and PSII (Munekage et al. 2002, 2004; Takahashi et al. 2009; Huang et al. 2011, 2012; Suorsa et al. 2012; Kono et al. 2014; Tikkanen and Aro 2014; Tikkanen et al. 2014, 2015; Chaux et al. 2015). Mature leaves have commonly been used for studying the role of CEF in regulation of photosynthetic electron flow and photosynthesis. However, little is known about the role of CEF in photosynthetic regulation in immature leaves that have relative lower ability to utilize light energy and thus are more susceptible to photoinhibition under high light.

At present, the specific mechanisms of CEF remain large controversies (for reviews, Leister and Shikanai 2013; Yamori and Shikanai 2016). Some studies indicated CEF plays an important role in CO_2 fixation under low light via ATP synthesis (Yamori et al. 2011; Nishikawa et al. 2012). However, Walker et al. (2014)

reported that CEF did not regulate ATP/NADPH under low light. Under high light, the CEF mutants of *Arabidopsis thaliana* and rice showed little change in CO_2 assimilation rate compared with wild types (Yamori et al. 2011; Nishikawa et al. 2012). However, theoretical analysis suggested that CEF responded to energy demand under high light (Walker et al. 2014). Our previous studies indicated that the main role of CEF differed under saturating and sub-saturating light intensities (Huang et al. 2015). Furthermore, the role of CEF under high light is dependent on the rates of CO_2 assimilation and photorespiration (Walker et al. 2014; Huang et al. 2015). In mature sun leaves, the relatively high rate of photorespiration probably needs more extra ATP from CEF. Meanwhile, PSI and PSII activities should be well protected against photoinhibition via activation of CEF. As a result, under high light CEF functions two different roles in mature sun leaves: one is to provide extra ATP and the other one is to favor photoprotection.

By comparison, in immature sun leaves, the relatively low rate of photosynthesis needs less extra ATP from CEF. On the other hand, the restriction of photosynthesis can increase the production of reactive oxygen species (ROS) (Takahashi and Murata 2005; Murata et al. 2007). It has been indicated that ROS not only inhibit the repair of photodamaged PSII at the step of protein synthesis (Nishiyama et al. 2001, 2006, 2011; Murata et al. 2007), but also accelerate the rate of PSII photodamage (Oguchi et al. 2009, 2011). Furthermore, photoinhibition of PSI is dependent on over-accumulation of ROS at the acceptor side of PSI (Hwang et al. 2004; Sonoike 2011). In order to prevent PSI and PSII from photodamage under condition of high light, immature leaves should have feasible mechanisms to regulate photosynthetic electron flow and diminish the production of ROS. It has been documented that CEF and NPQ are two important mechanisms to control the rate of ROS production. Previous studies indicated that immature leaves of *Arabidopsis thaliana* and tobacco showed significantly NDH-dependent CEF activity (Shikanai et al. 1998; Peng et al. 2009; Peng and Shikanai 2011). Furthermore, *pgr5* mutants of *A. thaliana* die at the seedling stage under fluctuating light conditions (Suorsa et al. 2016). These results suggest that CEF is essential for normal growth and photoprotection in immature

leaves. However, the physiological roles of CEF in immature leaves are not well known.

In plants, non-photochemical quenching (NPQ) is an important mechanism to diminish the production of ROS (Niyogi et al. 1997, 1998; Li et al. 2002). The activation of NPQ is dependent on the generation of ΔpH. It is well known that CEF-dependent building-up of ΔpH is essential for the activation of NPQ (Munekage et al. 2002, 2004; Takahashi et al. 2009; Kono et al. 2014; Tikkanen et al. 2015). Furthermore, under condition of excess light energy, another mechanism of CEF in photoprotection is to favor photosynthetic control via cytochrome b_6/f complex (Suorsa et al. 2012; Tikkanen and Aro 2014; Tikkanen et al. 2015). The CEF-dependent acidification of lumen under high light slows down the electron transfer via cytochrome b_6/f complex to PSI (Tikkanen and Aro 2014). This slowdown of electron transport to PSI enables PSI to function as a safe and efficient quencher of excitation energy (Shubin et al. 2008; Suorsa et al. 2012; Tikkanen et al. 2014). Or else, excess electron flow from PSII to PSI can lead to over-reduction of P700 and production of ROS at the acceptor side of PSI, which subsequently cause photodamage to PSI (Suorsa et al. 2012; Tikkanen et al. 2014). Photoinhibition of PSI is largely dependent upon the redox state of P700 reaction center (Sonoike 2011). Combination of over-reduction of P700 and high light easily induce severe photoinhibition of PSI (Munekage et al. 2004; Suorsa et al. 2012; Tikkanen et al. 2014). It has been indicated that PGR5-dependent CEF is essential for PSI photoprotection under conditions of high light and fluctuating light, due to the important roles of PGR5-dependent CEF in regulating P700 redox state and photosynthetic control (Munekage et al. 2004; Suorsa et al. 2012; Tikkanen et al. 2014). Therefore, taking into consideration the low ability of light use efficiency, we hypothesize that the main role of CEF in immature leaves is to protect PSI and PSII against photodamage.

In the present study, we determined the PSI and PSII activities, light response changes in PSII energy quenching and P700 redox state in immature and mature leaves from plants of *Erythrophleum guineense* grown in an open field. The aim of

this study is to examine the flexibility of CEF in regulation of photosynthetic electron flow in immature and mature leaves. The following questions were addressed: (1) How do immature leaves regulate photosynthetic electron flow to protect PSI and PSII against photoinhibition under high light? (2) Do the physiological roles of CEF in photosynthetic regulation differ between mature and immature leaves? Based on our results, the specific roles of CEF in photosynthetic regulation between immature and mature leaves were discussed.

Materials and methods

Plant materials and growth condition

Erythrophleum guineense G. Don (Fabaceae) is a large canopy species native to tropical Africa and produce high-quality timber. A few decades ago, *E. guineense* was introduced into Xishuangbanna tropical botanical garden, Chinese Academy of Science, and the plants exhibit good growth performance in the Xishuangbanna tropical botanical garden (21°54′ N, 101°46′ E) that is located in the northern boundary of the tropical zone. In the present study, immature and mature leaves of *E. guineense* raised in an open field were used for photosynthetic measurements. The highest irradiance on midday is up to 1,850 μmol photons m^{-2} s^{-1} in summer and 1,350 μmol photons m^{-2} s^{-1} in winter. The day/night temperatures are about 22/33ºC in summer and 12/21ºC in winter.

In the present study, mature leaves flushed three months ago and immature leaves flushed within one month were chosen for photosynthetic measurements. Twigs were cut from the trees and wrapped with wet tissue in black plastic bags for transport to the laboratory. Five minutes later, the twigs were inserted into buckets with water and the bottoms of these twigs were cut to avoid cavitation of stem. Subsequently, leaves were light-adapted and the in situ photosynthetic parameters were recorded.

Chlorophyll fluorescence and P700 measurements

Light response curves were monitored at 20ºC by simultaneously recording chlorophyll fluorescence and P700 redox state using the Dual PAM-100 (Heinz Walz,

Effeltrich, Germany). Six immature or mature sunlit leaves were light-adapted at 918 μmol photons m^{-2} s^{-1} for 20 min, and then light-adapted photosynthetic parameters were recorded after exposure to each light intensity (1804, 1450, 1173, 918, 754, 496, 325, 167, 89, 31 μmol photons m^{-2} s^{-1}) for 120 s.

The chlorophyll fluorescence parameters were calculated as follows: $F_v/F_m = (F_m - F_o)/F_m$, $F_o' = F_o/(F_v/F_m + F_o/F_m')$ (Oxborough and Baker 1997), qP = $(F_m' - F_s)/(F_m' - F_o')$, Y(II) = $(F_m' - F_s)/F_m'$ (Genty et al. 1989), Y(NPQ) = $F_s/F_m' - F_s/F_m$, Y(NO) = F_s/F_m (Hendrickson et al. 2004; Kramer et al. 2004). F_o and F_o' are the minimum fluorescence in the dark-adapted state and light-adapted state, respectively. F_m and F_m' are the maximum fluorescence after dark-adapted and light-adapted, respectively. F_o and F_m were determined after dark adaptation for 30 min. F_s is the light-adapted steady-state fluorescence.

The PSI photosynthetic parameters were measured by Dual PAM-100 based on P700 oxidation signal (difference of intensities of 830 and 875 nm pulse-modulated measuring light reaching the photodetector) (Klüghammer and Schreiber 2008), this method has been widely used in previous studies (Gao et al. 2011; Huang et al. 2011, 2012, 2013). The maximum photo-oxidizable P700 (P_m) was determined as described in previous studies (Huang et al. 2010a, b, 2016a; Tikkanen et al. 2014). The effective photochemical quantum yield of PSI, Y(I), was measured as $(P_m' - P)/P_m$. The PSI reaction center oxidation level, Y(ND), was calculated as P/P_m. The PSI reduction level, Y(NA), was measured as $(P_m - P_m')/P_m$.

Estimation of photosynthetic electron flow

Photosynthetic electron flow through PSI and PSII were calculated as: ETRII = Y(II) × PPFD × L_{abs} × 0.5, ETRI = Y(I) × PPFD × L_{abs} × 0.5, where 0.5 is the proportion of absorbed light reaching PSI or PSII, and L_{abs} is the absorptance (the fraction of the incident light absorbed by leaves). Because the chlorophyll content in the immature leaves was approximately 70% of the mature leaves (data not shown), the value of L_{abs} should be lower in the immature leaves. The value of CEF was estimated as ETRI – ETRII (Huang et al. 2012, 2015). It should be noted that the distribution of absorbed

light energy between PSI and PSII maybe differed in immature and mature leaves, which can lead to the possible impreciseness of ETRI, ETRII, and CEF.

Results

PSI and PSII activities

In the present study, the average leaf area for immature and mature leaves was 31.8 and 45.3 cm^2, respectively (Tab. 1). And the chlorophyll content per unit leaf area (SPAD) was 44.8 and 66.1 in immature and mature leaves, respectively (Tab. 1). The maximum quantum yield of PSII after dark-adaptation (F_v/F_m) was 0.72 and 0.83 in the studied immature and mature leaves of *E. guineense*, respectively (Tab. 1). Meanwhile, the maximum photo-oxidizable P700 (P_m) was 0.63 and 1.34 in the immature and mature leaves of *E. guineense*, respectively (Tab. 1). Based on these results, comparing to mature leaves, the relative values of F_v/F_m and P_m in the immature leaves were 87% and 47% in *E. guineense*, respectively. These results strongly suggested the juvenility of photosynthetic function in the immature leaves.

Light response changes in the steady-state PSI and PSII parameters

The effective quantum yield of PSI [Y(I)] gradually decreased with increasing light intensity, in accordance with previously presented results (Fig. 1A). Furthermore, under all light intensities, the immature leaves displayed significantly lower value of Y(I) than mature leaves. The light response change in the fraction of P700 that is oxidized by actinic light to the overall P700 [Y(ND)] was in accordance with previous results reported in wild-type plants. With the increase in light intensity, Y(ND) gradient increased (Fig. 1B). Interestingly, under all light intensities, values for Y(ND) were higher in immature leaves compared to mature leaves. The fraction of P700 that cannot be oxidized by a saturation pulse to the overall P700 [Y(NA)] was almost lower than 0.1 under all light intensities in both mature and immature leaves (Fig. 1C), indicating the prevention of over-reduction of PSI reaction centers.

Under all light intensities, the immature leaves had significantly lower values of effective quantum yield of PSII [Y(II)] than mature leaves in both species (Fig. 2A).

Meanwhile, the immature leaves displayed significantly higher quantum yield of regulated energy dissipation through non-photochemical quenching [Y(NPQ)] than mature leaves (Fig. 2B). The mature and immature leaves showed the similar values of quantum yield of non-regulated energy dissipation [Y(NO)] and the difference was not large (Fig. 2C). The immature leaves displayed higher PSII acceptor side limitation than mature leaves, as indicated by the higher value of the proportion of closed PSII reaction centers (1 – qP) in immature leaves (Fig. 3A). However, the immature leaves had higher capacity of non-photochemical quenching (NPQ) than mature leaves (Fig. 3B), indicating the higher capacity of thermal energy dissipation in the immature leaves. Furthermore, the light saturating point of NPQ in immature leaves was much lower than mature leaves, suggesting the stronger acidification of lumen under low and moderate light intensities. The similar relationship between 1 – qP and Y(ND) suggested the symmetrical accumulation of redox poises of PSII acceptor side and PSI donor side (Fig. 4), indicating that the rate limiting step of the electron transport is between PSII and PSI. It supports the role of PGR5-dependent CEF in regulation of photosynthetic electron transport via the Cyt b_6/f (Suorsa et al. 2012; Tikkanen and Aro 2014; Zivcak et al. 2014).

Light response changes in photosynthetic electron flow

In higher plants, the NDH and PGR5 pathway explained most of the CEF judging from the phenotype of double mutants of *Arabidopsis thaliana*. The P700 redox state under high light is mainly regulated by PGR5-dependent pathway but not by NDH-dependent pathway (Munekage et al. 2004; Kou et al. 2015). The high level of P700 oxidation ratio under high light, as indicated by the value of Y(ND) (Fig. 1B), suggested the activation of PGR5-dependent CEF in both immature and mature leaves of *E. guineense*.

Light response changes in electron transport through PSI (ETRI), electron transport through PSII (ETRII), and CEF largely differed between the immature and mature leaves in both species. The immature leaves had much lower capacities of photosynthetic electron flow than mature leaves (Fig. 5). Furthermore, in the

immature leaves, values for ETRI, ETRII, and CEF reached the maximum at a low light of 167 μmol photons m^{-2} s^{-1}. However, in mature leaves, ETRI, ETRII, and CEF reached the maximum at a high light of approximately 754 μmol photons m^{-2} s^{-1} (Fig. 5). In *E. guineense*, the maximum values for ETRI, ETRII, and CEF were approximately 111, 69, and 42 μmol electrons m^{-2} s^{-1} in mature leaves. By comparison, the maximum values for ETRI, ETRII, and CEF were approximately 35, 18, and 17 μmol electrons m^{-2} s^{-1} in the immature leaves.

Although CEF-dependent generation of ΔpH plays an important role in the activation of NPQ, the relationship between CEF activation and NPQ values differed between mature and immature leaves (Fig. 6). The same value of NPQ was accompanied with much higher CEF activation in the mature leaves. In the other words, the same level of lumen acidification was accompanied with higher CEF activity in mature leaves. Therefore, the fraction of CEF-dependent generation of ΔpH that contributes to lumen acidification was much lower in the mature leaves. The major role of CEF-dependent generation of ΔpH was to induce lumen acidification in the immature leaves.

Discussion

Cyclic electron flow around photosystem I has been documented as an essential mechanism for photosynthetic regulation and photoprotection (Munekage et al. 2002; Takahashi et al. 2009; Tikkanen and Aro 2014). The CEF-dependent formation of ΔpH can be relaxed mainly through two different pathways: (1) ATP synthesis via ATP synthase, and (2) thermal energy dissipation through xanthophylls cycle. Previous studies on the physiological roles of CEF mainly focus on mature leaves, and the specific roles of CEF in immature leaves are little known. Here, we compared the roles of CEF in regulation of photosynthesis in both immature and mature leaves of *E. guineense*. Surprisingly, although the immature leaves had a much lower CEF activity than mature leaves, immature leaves display a similar/higher capacity of NPQ compared to mature leaves. Under high light, 80% of NPQ activation is based on the CEF-dependent acidification of thylakoid lumen (Sato et al. 2014). These results

suggested that the specific roles of CEF in photosynthetic regulation differed between the immature and mature leaves. In immature leaves, the CEF-dependent generation of ΔpH mainly functions for photoprotection. In detail, an increased steady-state proton motive force principally results in stronger lumen acidification, which in turn enhances dissipation of excess energy and photosynthetic control. As a result, PSI and PSII can be well protected under high light in immature leaves that have lower light use efficiency. By comparison, in mature leaves, the large proportion of CEF-dependent generation of ΔpH is relaxed via ATP synthase and leading to extra ATP synthesis, and the small proportion contributes to photoprotection via lumen acidification.

Characteristics of photosynthetic parameters in immature leaves

Results indicated that the immature leaves displayed significantly lower PSI and PSII activities, ETRI, ETRII, and CEF than mature leaves. In the immature leaves, the relative values of F_v/F_m and P_m in the immature leaves were 87% and 47% in *E. guineense*, respectively, when compared to mature leaves (Tab. 1). The maximum of ETRI, ETRII and CEF in immature leaves were 32%, 26% and 40% of mature leaves in *E. guineense*, respectively (Fig. 5). Taking together, the relative capacity of photosynthetic electron flow in the immature leaves was more similar to P_m rather than F_v/F_m. Therefore, PSI activity is an important limiting factor for photosynthetic electron flow during leaf development. The photosynthetic capacity can be limited by many components involved in both light and dark reactions, such as PSI (Zivcak et al. 2015), PSII (Tikkanen et al. 2014), Rubisco (Hudson et al. 1992), glyceraldehyde-3-phosphate dehydrogenase (Price et al. 1995), Cyt b_6/f and ATP synthase (Rott et al. 2011; Yamori et al. 2011). Comparing to the *in vitro* biochemical measurements, P_m is an easily measured photosynthetic parameter that can be used as an indicator of the maturity of leaf development.

Role of CEF in the immature and mature leaves

Our present study indicated that the immature leaves displayed highly NPQ values

under high light. However, these high NPQ values in the immature leaves were not caused by high CEF activity. Actually, immature leaves displayed much lower CEF activity than mature leaves. As a result, a low CEF activity can lead to a high NPQ value in the immature leaves. This result, in some respects, is contrary to the conclusions of previous studies on CEF in mature leaves. In higher plants, the CEF-dependent formation of ΔpH via PGR5 pathway is essential for the normal activation of NPQ under high light, judging from the phenotype of *pgr5* plants of *Arabidopsis thaliana* (Munekage et al. 2002, 2004; Takahashi et al. 2009; Suorsa et al. 2012; Kono et al. 2014; Tikkanen et al. 2015). Stimulation of CEF activity via over-expressing ferredoxin enhances NPQ in mature leaves of transplastomic tobacco (Yamamoto et al. 2006). Leaves grown under high light dissipated the excess light energy through NPQ by enhancing the CEF activity (Miyake et al. 2005; Huang et al. 2015). In mature leaves, PSI photoinhibition significantly affects CEF activity and thus depresses NPQ (Huang et al. 2013; Brestic et al. 2015; Zivcak et al. 2015). These previous studies suggested that in mature leaves a high CEF activity is a prerequisite for a high capacity of NPQ. Non-photochemical quenching is induced by a high ΔpH that is generated by photosynthetic electron transport. The acidification of thylakoid lumen activates qE by protonating the protein PsbS (Li et al. 2002) and by activating violaxanthin deepoxidase, which converts violaxanthin to antheraxanthin and zeaxanthin in the xanthophyll cycle (Demmig-Adams and Adams III 1996). As a result, NPQ is a fluorescence indicator of lumen acidification (Zivcak et al. 2014). Under high light, 80% of NPQ induction was based on CEF-dependent lumen acidification by generating ΔpH (Sato et al. 2014). When illuminated at a strong light of 1804 μmol photons m^{-2} s^{-1}, the mature leaves had approximately 2.5 fold CEF activity but similar NPQ compared to the immature leaves, indicating that the fraction of CEF-dependent ΔpH formation that induces NPQ via lumen acidification largely differed between the mature and immature leaves.

The observation that high amounts of NDH subunits are present in etioplasts and immature leaves (Fischer et al. 1997; Guera et al. 2000; Nixon 2000; Peltier and Cournac 2002), led to the hypothesis that 'chlororespiratory' components may be

essential for chloroplast biogenesis via energy transduction. However, the absence of any obvious phenotype related to greening in NDH-deficient transformants suggests that this role is not essential (Rumeau et al. 2007). As a result, the contribution of NDH-CEF to ATP synthesis in immature leaves is assumed to be low. Furthermore, immature leaves had lower ability to utilize light energy, as indicated by the low values of ETRII. Accordingly, the ATP demand for primary metabolism in immature leaves should be much lower than mature leaves, and the ability of dissipation of excess light energy is critical for photoprotection. It has been indicated that ATP synthase repression results in increased lumen acidification and accelerated induction of photoprotective mechanisms (Rott et al. 2011). Once the efflux of proton via ATP synthase was restricted under high light and drought stress, the lumen acidification of thylakoid increased to dissipate excess light energy, due to activation of CEF (Miyake et al. 2005; Huang et al. 2012; Zivcak et al. 2014). Here, the higher NPQ values under high light in the immature leaves suggested stronger lumen acidification. In addition, the linear relationship between PSII acceptor side and PSI donor side (Fig. 5) supported the CEF-dependent photosynthetic control via the cytochrome b_6/f. Therefore, in the immature leaves, CEF-dependent formation of ΔpH principally contributes to lumen acidification of thylakoid, which subsequently functions for photoprotection via NPQ, photosynthetic control, and regulating P700 redox state.

In mature leaves, higher rates of the Calvin cycle and photorespiration require a proper ATP/NADPH ratio approximately being 1.6. Meanwhile, the stoichiometry of the ATP/NADPH ratio produced by LEF (electron flow from H_2O to $NADP^+$) is thought to be 1.29 (Edwards and Walker 1983). Such a change in energy demand necessitates a flexible mechanism to provide extra ATP synthesis and then balance that ratio. In addition to LEF, the CEF-dependent generation of ΔpH can help ATP synthesis via ATP synthase (Shikanai 2007; Kramer and Evans 2011; Yamori et al. 2011; Huang et al. 2015; Wang et al. 2015). As a result, an important role of CEF in mature leaves is to help extra ATP synthesis and regulate ATP/NADPH ratio. Our results showed that the mature leaves had approximately 2.5 times CEF activity than the immature leaves. Meanwhile, the level of lumen acidification was similar/lower in

the mature leaves (as indicated by NPQ values). Therefore, it is conceivable that a large proportion of CEF-dependent formation of ΔpH was relaxed to produce extra ATP, and the lumen acidification was induced by the small proportion of CEF. Lumen acidification not only activates NPQ, but also controls photosynthetic rate. Over-acidification of thylakoid lumen would cause depressions of linear electron flow and photosynthesis. In mature leaves, the high rate of photosynthetic CO_2 assimilation needs relatively high rate of LEF. As a result, over-acidification of thylakoid lumen should be prevented to optimize photosynthesis. If the higher CEF activity induced over-acidification of thylakoid lumen, the mature leaves cannot maintain optimal carbon gain via photosynthetic CO_2 assimilation. Thus, the specific role of CEF-dependent ΔpH in regulation of photosynthesis largely depends on the rate of photosynthesis.

Photoprotection in immature leaves

For the immature leaves, light saturating point of ETRII was much lower than that of mature leaves (Fig. 6B). Restriction of the Calvin cycle can accelerate production of ROS that inhibit the repair of PSII from photodamage. (Takahashi and Murata 2005; Murata et al. 2007). To control the production of ROS, immature leaves have to activate thermal energy dissipation strongly. Under conditions in which absorbed light is in excess of the requirements for photosynthesis, the activation of CEF can increase the level of lumen acidification, which causes the major light chlorophyll a/b light harvesting antenna protein (LHCII) to dissipate excess excitation energy harmlessly as heat. Subsequently, the energy transfer efficiency from LCHII to the photosystems is reduced (Tikkanen and Aro 2014), which then decreases ROS production and favors the repair of PSII. The higher NPQ capacity in the immature leaves than mature leaves suggested that NPQ played an important role in diminishing the production of ROS and alleviating PSII photoinhibition. Furthermore, acidification of the lumen could drive a Ca^{2+}/H^+ antiport to sequester Ca^{2+} in the lumen (Ettinger et al. 1999), which could stabilize the oxygen-evolving complex against photodamage (Krieger and Weis 1993). Impairment of PGR5-dependent CEF largely accelerates the

rate of photodamage to PSII (Takahashi et al. 2009). It is conceivable that the CEF-dependent generation of ΔpH probably suppresses the rate of photodamage to PSII by protecting the oxygen-evolving complex (Takahashi et al., 2009; Huang et al. 2016b). Therefore, the rate of photodamage to PSII in the immature leaves may be controlled by CEF activation via lumen acidification.

Because PSI tends to be damaged when electron flow from PSII to PSI exceeds the capacity of PSI electron acceptors (Suorsa et al., 2012; Tikkanen et al., 2014), the rise in ΔpH due to CEF activation under saturating light conditions can control the electron flow from PSII to PSI via the Cyt b_6/f complex, thereby protecting PSI against photodamage (Suorsa et al., 2012; 2013; Tikkanen and Aro 2014; Tikkanen et al., 2015). The pH-dependent control of the intersystem electron transport has been proposed to be an important mechanism for short-term regulation of electron transport (Tikhonov 2015). The immature leaves showed symmetrical accumulation of PSII acceptor side and PSI donor side (Fig. 5), indicating that the rate limiting step of the electron transport is between PSII and PSI. This result supported the role of PGR5-dependent CEF in regulation of photosynthetic electron transport via the cytochrome b_6/f. Furthermore, over-reduction of the PSI acceptor side under high light can lead to severe PSI photodamage in *pgr5* plants of *Arabidopsis thaliana* (Munekage et al., 2002; Suorsa et al., 2012; Kono et al., 2014; Tikkanen et al., 2014). In wild-type of *A. thaliana*, CEF activation can prevent over-reduction on the PSI acceptor side by increasing the P700 oxidation ratio. In our present study, the immature leaves displayed significantly higher P700 oxidation ratio than mature leaves, as indicated by the data of Y(ND) (Fig. 2C, D). What is more, over-reduction of P700 was prevent in immature leaves, as indicated by the low values of Y(NA) (Fig. 2E, F). These results suggested that in immature leaves CEF prevented PSI from photodamage via photosynthetic control and regulating P700 redox poise.

Conclusion

In the present study, we examined the specific roles of CEF in photosynthetic regulation in immature and mature leaves. In mature leaves, a large proportion of

CEF-dependent generation of ΔpH contributes to ATP synthesis which regulates ATP/NADPH ratio and optimizes photosynthetic CO_2 assimilation and photosynthetic electron flow. Meanwhile, a relative small proportion of CEF-dependent generation of ΔpH favors photoprotection. By comparison, in immature sun leaves, the ability of leaves to utilize light energy is restricted due to lower photosynthetic activity. To protect PSI and PSII against photodamage, CEF-dependent generation of ΔpH primarily enhances lumen acidification, which then activates NPQ, regulates P700 redox state and controls the transfer of electrons via the Cyt b_6/f complex. As a result, the immature leaves have similar/higher capacity of NPQ despite of much lower CEF activity. These findings highlight the different roles of CEF in photosynthetic regulation between immature and mature leaves.

Statistical analysis

All results were displayed as mean values from six independent experiments. The data were subjected to one-way ANOVA using SPSS 16.0 software. Statistically significant differences between immature and mature leaves were examined with T-test (α = 0.05).

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Disclosures

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contribution

W.H. and S.B.Z. designed study. W.H. performed all experiments. W.H. and Y.J.Y. analyzed data. W.H. wrote manuscript with significant input from Y.J.Y. and S.B.Z.

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Figure legends

Figure 1

Light responses of complementary quantum yields of PSI recorded in immature and mature leaves of *Erythrophleum guineense*. Y(I), the effective quantum yield of PSI; Y(ND), the fraction of overall P700 that is oxidized in a given state; Y(NA), the fraction of overall P700 that cannot be oxidized in a given state. The light response curves were obtained after previous induction at 918 μmol photons m^{-2} s^{-1} for 20 minutes, and the duration of each interval with a given light intensity was 120 s. Values are means ± SE (n = 6). Asterisks indicate significant differences between mature and immature leaves.

Figure 2

Light responses of complementary quantum yields of PSII recorded in immature and mature leaves of *Erythrophleum guineense*. Y(II), the effective quantum yield of PSII; Y(NPQ), the quantum yield of regulated non-photochemical quenching in PSII; Y(NO), the fraction of energy captured by PSII passively dissipated in form of heat and fluorescence. These light response curves were obtained simultaneously with Fig. 1. Values are means ± SE (n = 6). Asterisks indicate significant differences between mature and immature leaves.

Figure 3

Light response changes in the redox poise of the primary electron acceptor of PSII (1 – qP) (**A**), and non-photochemical quenching of PSII (NPQ) (**B**) in immature and mature leaves of *Erythrophleum guineense*. The light response curves were obtained according to the method described in Fig. 1. Values are means ± SE (n = 6). Asterisks indicate significant differences between mature and immature leaves.

Figure 4

The relationship between the redox poises of PSII and PSI derived from the simultaneous chlorophyll fluorescence and P700 records under various light

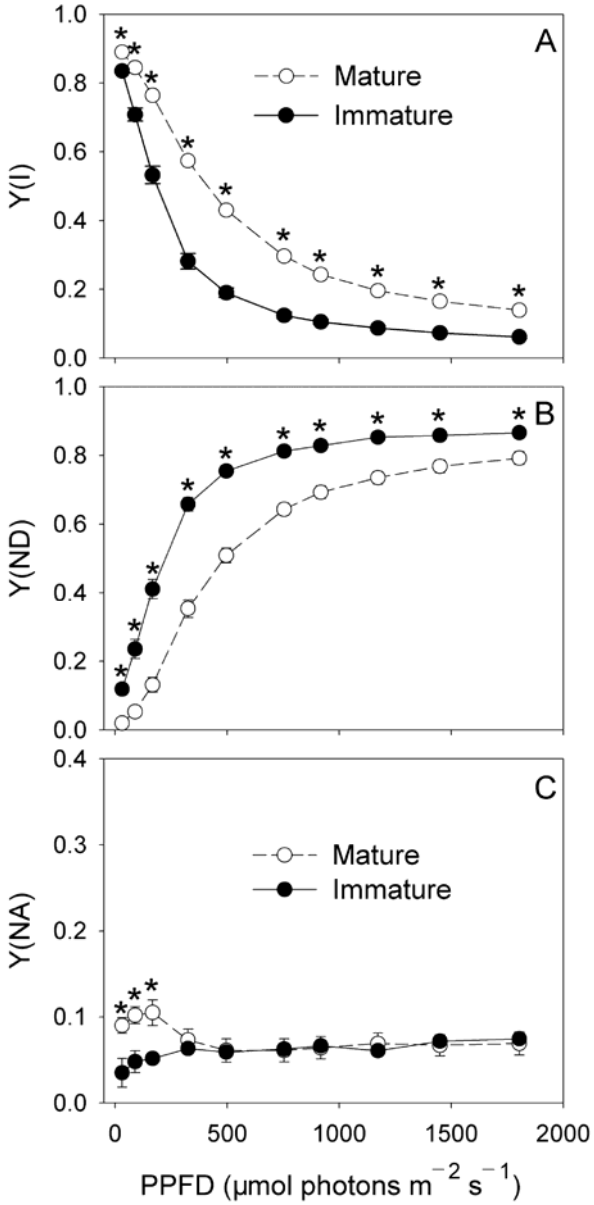
intensities in immature and mature leaves of *Erythrophleum guineense*. Light response data in Figures 1 and 3 were used.

Figure 5

Light response changes in photosynthetic electron flow in immature and mature leaves of *Erythrophleum guineense*. L_{abs} represents the fraction of the incident light absorbed by leaves. ETRI, electron transport rate through PSI; ETRII, electron transport rate through PSII; CEF, rate of cyclic electron flow around PSI. The light response curves were obtained according to the method described in Fig. 1. Values are means ± SE (n = 6). Asterisks indicate significant differences between mature and immature leaves.

Figure 6

The relationship between the CEF activation and NPQ values derived from the simultaneous chlorophyll fluorescence and P700 records under various light intensities in immature and mature leaves of *Erythrophleum guineense*. Light response data in Figures 3B and 5C were used. The calculation of CEF is based on assumption of L_{abs} being 0.85 and 0.7 in mature and immature leaves, respectively.



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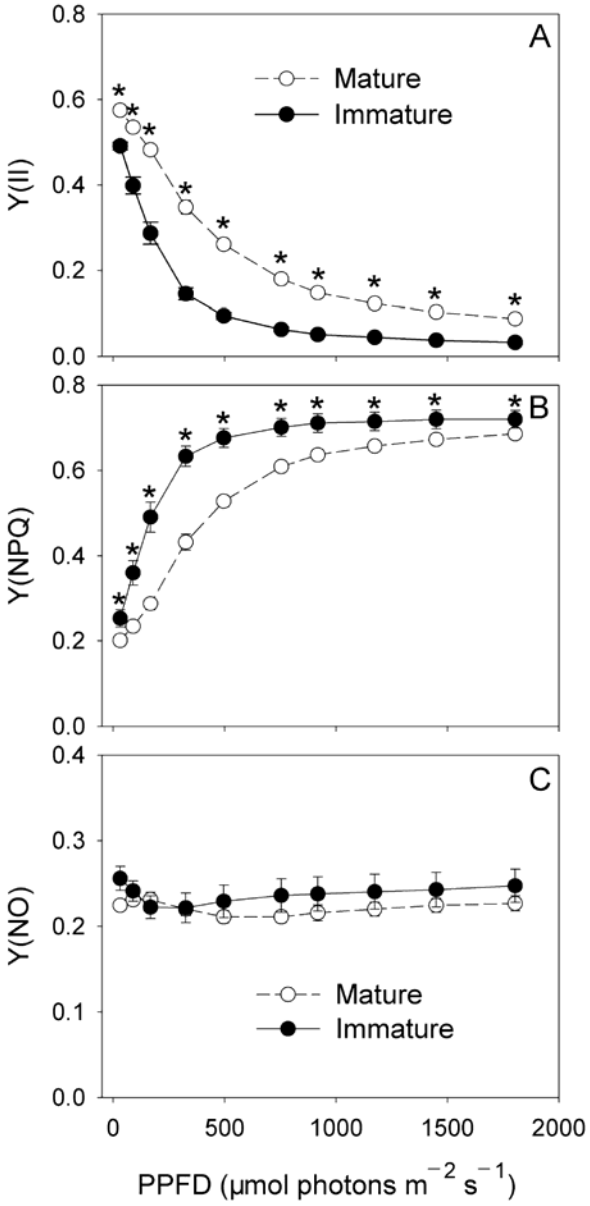

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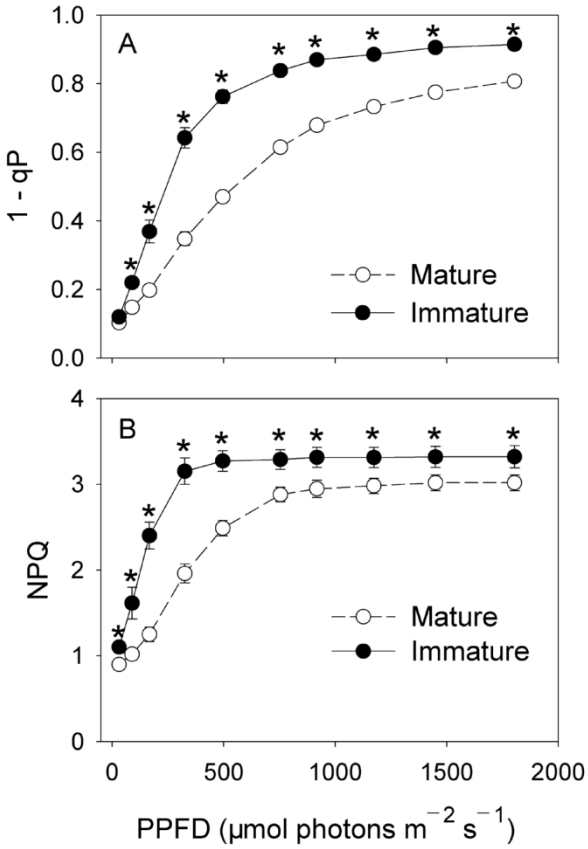

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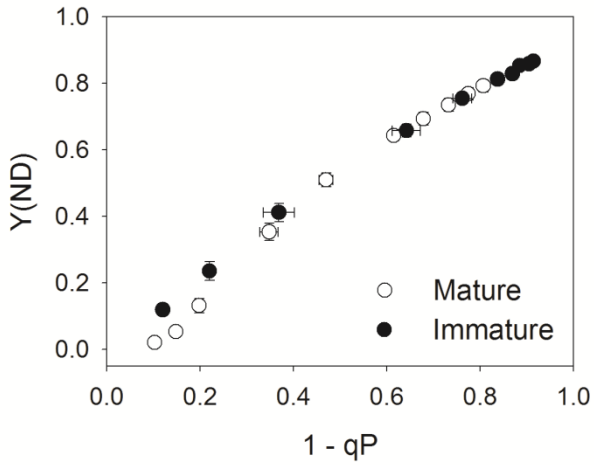

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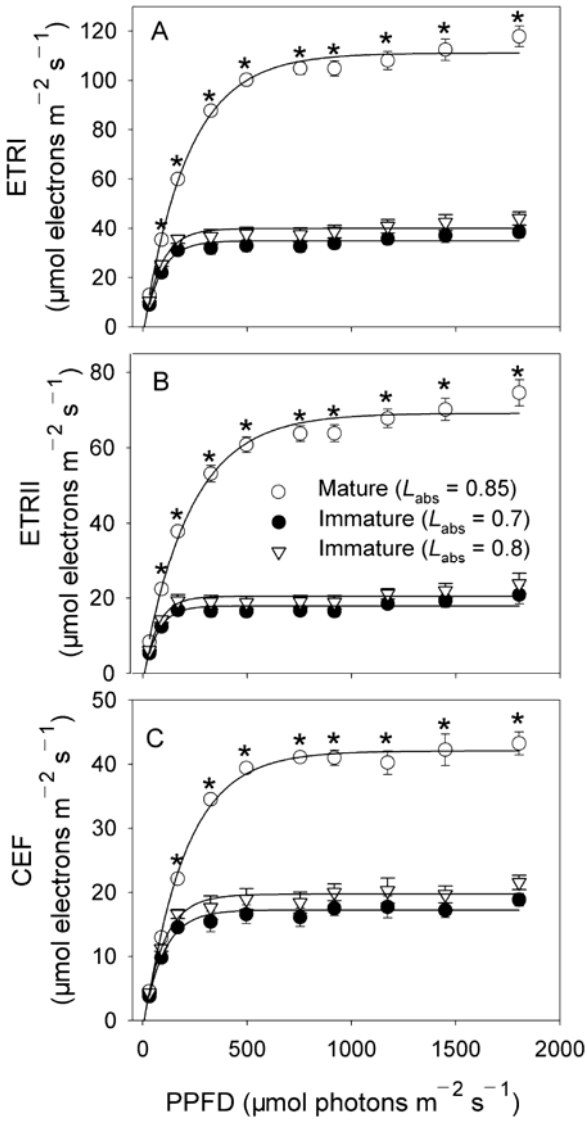

Figure 5. Light response changes in photosynthetic electron flow in immature and mature leaves of *Erythrophleum guineense*. L_{abs} represents the fraction of the incident light absorbed by leaves. ETRI, electron transport rate through PSI; ETRII, electron transport rate through PSII; CEF, rate of cyclic electron flow around PSI. The light response curves were obtained according to the method described in Fig. 1. Values are means ± SE (n = 6). Asterisks indicate significant differences between mature and immature leaves.

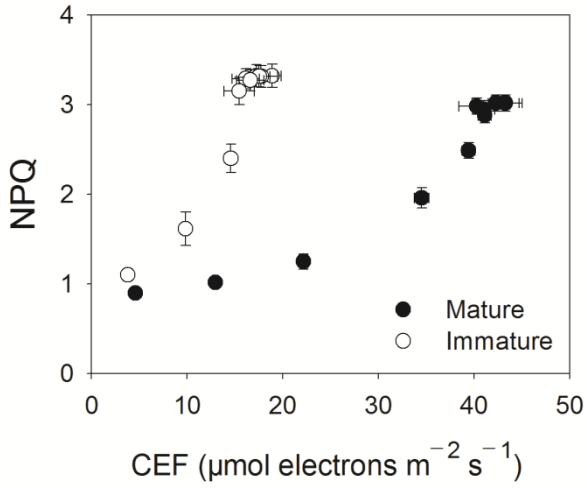

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Table 1. Leaf area, chlorophyll content, PSI and PSII activity for the immature and mature leaves of *E. guineense*. Values are means ± SE (n = 6). The different letters indicate significant differences between mature and immature leaves.

Parameters	Immature leaves	Mature leaves
Leaf area (cm^2)	31.8 ± 1.4a	45.3 ± 1.3b
Chlorophyll content (SPAD)	44.8 ± 0.76a	66.1 ± 0.51b
Fv/Fm	0.72 ± 0.004a	0.83 ± 0.001b
Pm	0.63 ± 0.05a	1.34 ± 0.09b