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ORIGINAL ARTICLE

In vivo regulation of thylakoid proton motive force in immature leaves

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Abstract

In chloroplast, proton motive force (pmf) is critical for ATP synthesis and photoprotection. To prevent photoinhibition of photosynthetic apparatus, proton gradient (ΔpH) across the thylakoid membranes needs to be built up to minimize the production of reactive oxygen species (ROS) in thylakoid membranes. However, the regulation of thylakoid pmf in immature leaves is little known. In this study, we compared photosynthetic electron sinks, P700 redox state, non-photochemical quenching (NPQ), and electrochromic shift (ECS) signal in immature and mature leaves of a cultivar of Camellia. The immature leaves displayed lower linear electron flow and cyclic electron flow, but higher levels of NPQ and P700 oxidation ratio under high light. Meanwhile, we found that pmf and ΔpH were higher in the immature leaves. Furthermore, the immature leaves showed significantly lower thylakoid proton conductivity than mature leaves. These results strongly indicated that immature leaves can build up enough ΔpH by modulating proton efflux from the lumenal side to the stromal side of thylakoid membranes, which is essential to prevent photoinhibition via thermal energy dissipation and photosynthetic control of electron transfer. This study highlights that the activity of chloroplast ATP synthase is a key safety valve for photoprotection in immature leaves.

Keywords Chloroplast ATP synthase · Electron transfer · Immature leaves · Lumenal acidification · P700 redox state · Photoprotection

Abbreviations

CEF	Cyclic electron flow around photosystem I;
ΔpH	Proton gradient across the thylakoid membranes
g_{H}^{+}	Thylakoid proton conductivity
LEF	Linear electron flow
NPQ	Non-photochemical quenching
pmf	Proton motive force
qL	The fraction of open PSII reaction centers based on the "lake model" of PSII antenna pigment organization
Y(I)	Quantum yield of PSI photochemical quenching
Y(II)	Effective quantum yield of PSII photochemistry
Y(NA)	The quantum yield of PSI non-photochemical energy dissipation due to the acceptor-side limitation
Y(ND)	The quantum yield of PSI non-photochemical energy dissipation due to the donor-side limitation

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Introduction

Leaves absorb light energy to drive photosynthetic electron flow through the thylakoid membranes in chloroplasts. In linear electron flow (LEF), 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, protons are released by water splitting in PSII and the quinone cycle in the Cyt b_6/f complex, generating proton motive force (pmf) across the thylakoid membranes. During cyclic electron flow (CEF), electrons from either NADPH or ferredoxin are cycled back from PSI to the plastoquinone pool, which is coupled to the generation of a proton gradient across the thylakoid membranes (ΔpH) but does not lead to the reduction of $NADP^+$ (Johnson 2011). The pmf is energetically composed of two components, ΔpH and a membrane potential (ΔΨ) (Cruz et al. 2001). Both ΔpH and ΔΨ drive the synthesis of ATP via the chloroplast ATP synthase (Kramer et al. 2003, 2004; Avenson et al. 2005), but the ΔpH component has additional impact on regulating light capture and electron transfer (Ruban et al. 2007; Takizawa et al. 2007; Tikkanen and Aro 2014). In mature leaves of higher plants, ΔpH plays an essential role in photosynthetic regulation

responding to environmental stresses (Munekage et al. 2002, 2004; Suorsa et al. 2012, 2016; Wang et al. 2015; Yamori et al. 2016). However, the regulation of pmf in immature leaves is less well understood.

In immature sun leaves, the combined effects of biochemical and structural limitations lead to the relatively low rate of photosynthesis (Niinemets et al. 2012). As a result, the imbalance between the capacity for light capture and the rate of carbon assimilation increases the risk of production of reactive oxygen species (ROS) (Takahashi and Murata 2005; Miller et al. 2007; Murata et al. 2007; Takahashi et al. 2007; Schmitt et al. 2014). 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; Allakhverdiev et al. 2005; Mohanty et al. 2007; Murata et al. 2007), but also accelerate the rate of PSII photodamage (Oguchi et al. 2009, 2011; Krieger-Liszkay et al. 2011; Vass 2011). Furthermore, ROS can cause photoinhibition of PSI (Hwang et al. 2004; Sonoike 2011; Takagi et al. 2016). PSI plays critical roles for photoprotection, photosynthetic electron flow, and plant growth (Zivcak et al. 2015; Brestic et al. 2015; Yamori et al. 2016). In order to prevent PSI and PSII from photodamage under high light conditions, immature leaves should have feasible mechanisms to diminish the production of ROS within the thylakoid membranes or in the chloroplast stroma. Indeed, it has been shown that pea etioplasts accumulate a variety of antioxidant proteins, such as ascorbate peroxidase and glutathione reductase (Kanervo et al. 2008). The acidification of lumen under high light slows down the electron transfer to PSI via the Cyt b_6f complex (Tikkanen and Aro 2014). This slowdown of electron transport enables PSI to function as a safe and efficient quencher of excitation energy (Shubin et al. 2008; Suorsa et al. 2012, 2016; Tiwari et al. 2016). Otherwise, excess electron flow from PSII to PSI can lead to the production of ROS within PSI, which subsequently cause photodamage to PSI (Suorsa et al. 2012; Tikkanen et al. 2014; Kanazawa et al. 2017; Takagi et al. 2017). Therefore, taking into consideration the importance of lumen acidification in photoprotection, immature leaves should have feasible mechanisms to maintaining high levels of lumen acidification.

The extent of thylakoid lumen acidification is determined by two factors: (1) the proton influx via LEF and CEF, and (2) thylakoid proton conductivity via ATP synthase. The extent of lumen acidification is a net result of the proton influx and the proton efflux. Under environmental stresses such as drought, high light, and fluctuating light, the activation of CEF probably enhances the formation of ΔpH in wild-type plants (Suorsa et al. 2012, 2016; Zivcak et al. 2013, 2014; Kono et al. 2014; Wang et al. 2015). In *pgr5 Arabidopsis* mutants, the proton influx is partly impaired due to the decreased CEF activity, and the higher proton conductivity further leads to the failing of lumen acidification (Avenson et al. 2005; Wang et al. 2015; Suorsa et al. 2016), causing severe photoinhibition of PSI and PSII (Munekage et al. 2002; Takahashi et al. 2009; Suorsa et al. 2012; Tikkanen et al. 2014; Yamori et al. 2016). Therefore, the coordination between thylakoid proton influx and proton efflux must be finely regulated. Recently, we found that under high light immature leaves have relatively lower CEF activity than mature leaves (Huang et al. 2017a), suggesting a low proton influx activity in immature leaves. In order to maintain a sufficient lumen acidification, the thylakoid proton efflux should be downregulated. Therefore, we hypothesize that the lower proton conductivity through ATP synthase is critical for maintaining optimal lumen acidification in immature leaves.

Non-photochemical quenching (NPQ) acts as a photoprotective mechanism by dissipating excess excitation energy from the light-harvesting complexes to prevent over-excitation of PSII (Li et al. 2002; Ruban et al. 2007; Takahashi et al. 2009). It has been documented that the activation of NPQ is largely based on the formation of ΔpH across the thylakoid membranes (Munekage et al. 2002; Suorsa et al. 2012, 2016; Sato et al. 2014). In mature leaves illuminated at high light, a higher NPQ is usually accompanied with a higher CEF activity (Miyake et al. 2005; Yamamoto et al. 2006). Recently, we found that the immature leaves displayed similar NPQ capacity to the mature leaves level under high light, whereas immature leaves displayed much lower CEF activity than mature leaves (Huang et al. 2017a). Therefore, the regulation of pmf in immature leaves should be different from mature leaves.

In this study, we determined the photosynthetic electron sinks, P700 redox state, NPQ, and ECS signal in immature and mature leaves from plants of a *Camellia* cultivar "Yulinjia." The aim of this study is to understand the regulation of pmf in immature leaves. The following question was addressed: Whether immature leaves depress the thylakoid proton conductivity to compensate for the deficiency of proton influx activity? Based on our results, the role of ATP synthase in photoprotection in immature leaves is discussed.

Materials and methods

Plant materials

In the present study, three-year-old plants of a Camellia cultivar "Yulinjia" were used for photosynthetic measurements. Plants were grown in a greenhouse with light condition of 60% sunlight (with maximum mid-day light intensity of approximately 1200 μmol photons m^{-2} s^{-1}). The plants were watered and fertilized normally, thus neither drought nor nutrition stress were experienced. This species has a leaf life span longer than 2 years, and the maturation of leaves needs

at least 3 months. Fully expanded leaves that had flushed one-year ago and young leaves flushed within 1 month were chosen to represent mature and immature leaves, respectively, in this study.

Gas exchange measurement

Gas exchange was monitored synchronously using Li-6400XT (LI-COR Biosciences, Lincoln, NE, USA) and a 2-cm^2 measuring head (6400-40 Leaf Chamber Fluorometer; LI-COR Biosciences). During measurements, relative air humidity and air temperature were approximately 60% and 25 °C, respectively. The atmospheric CO_2 concentration was controlled at 400 µmol mol^{-1} by the Li-6400XT. When leaves displayed steady-state high levels of g_s and photosynthetic rate after light adaptation by natural sunlight, the rates of CO_2 assimilation at 1500 µmol photons m^{-2} s^{-1} were recorded after 3 min adaptation.

Chlorophyll fluorescence and P700 measurements

Light response curves were monitored at 20 °C 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 first light-adapted at 923 µmol photons m^{-2} s^{-1} for 20 min, and next light-adapted photosynthetic parameters were recorded after exposure to each light intensity (1450, 918, 606, 325, 167, and 54 µmol photons m^{-2} s^{-1}) for 2 min.

The chlorophyll fluorescence parameters were calculated as follows: $F_v/F_m = (F_m - F_o)/F_m$, $Y(II) = (F_m' - F_s)/F_m'$ (Genty et al. 1989), $NPQ = (F_m - F_m')/F_m'$, $qL = (F_m' - F_s)/(F_m' - F_o') \times F_o'/F_s$, $F_o' = F_o/(F_v/F_m + F_o/F_m')$ (Oxborough and Baker 1997). 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 adaptation and light adaptation, respectively. F_s is the light-adapted steady-state fluorescence. F_o and F_m were determined after dark adaptation for 30 min. F_v/F_m was recorded to estimate the PSII activity. Y(II) is the effective quantum yield of PSII; NPQ indicates the non-photochemical quenching in PSII; and qL represents the fraction of open PSII reaction centers based on the "lake model" of PSII antenna pigment organization. The chlorophyll fluorescence-derived LEF was calculated as $LEF = PPFD \times Y(II) \times 0.84 \times 0.5$.

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), a method that has been widely used in recent studies (Huang et al. 2011, 2012; Kono et al. 2014; Suorsa et al. 2016; Takagi et al. 2016, 2017). The $P700^+$ signals (P) may vary between a minimal (P700 fully reduced) and a maximal level (P700 fully oxidized). The maximum level (P_m) was determined with application of a saturation pulse (300 ms and 10,000 µmol photons m^{-2} s^{-1}) after pre-illumination with far-red light. P_m' was determined similar to P_m but with actinic light instead of far-red light. P_m was recorded to estimate the PSI activity (Huang et al. 2010a, b; Suorsa et al. 2012; Tikkanen et al. 2014; Yamori et al. 2016). The quantum yield of PSI was calculated as $Y(I) = (P_m' - P)/P_m$. The P700 oxidation ratio in a given actinic light was calculated as $Y(ND) = P/P_m$, and the fraction of P700 that cannot be oxidized by saturation pulse to the overall P700 was calculated as $Y(NA) = (P_m - P_m')/P_m$.

Electrochromic shift analysis

The ECS signal was monitored as the absorbance change at 515 nm by using a DUAL PAM-100 (Walz, Effeltrich, Germany) equipped with a P515/535 emitter-detector module (Walz). Leaves were first dark adapted for 30 min and then the 515-nm absorbance change induced by a single turnover flash (ECS_{ST}) was measured. Afterwards, leaves were illuminated at 606 µmol photons m^{-2} s^{-1} for 15 min to activate the electron sink in photosynthesis, and then the light intensity dependence of ECS signal was obtained after 5 min of illumination at each actinic light (1450, 918, 606, 325, and 167 µmol photons m^{-2} s^{-1}). The analysis of ECS dark-interval relaxation kinetics ($DIRK_{ECS}$) was done by the method of Sacksteder et al. (2001) and Takizawa et al. (2008). The difference in total pmf between light and dark, ECS_t, was estimated from the total amplitude of the rapid decay of the ECS signal during the dark pulse. All ECS_t levels were normalized against the magnitude of ECS_{ST}. This normalization accounted for changes in leaf thickness and chloroplast density between leaves (Takizawa et al. 2008; Livingston et al. 2010; Wang et al. 2015; Takagi et al. 2017). The slow relaxation of ECS signal allowed the contributions of proton gradient (ΔpH) and the electrochemical gradient (ΔΨ) across the thylakoid membranes to be delineated (Sacksteder and Kramer 2000; Cruz et al. 2005). It should be noted that Johnson and Ruban (2014) questioned the use of ECS signals to estimate ΔpH and ΔΨ, although the division of pmf into ΔpH and ΔΨ largely improved our understanding of the photosynthetic regulation under environmental stresses. The magnitudes of ΔpH and ΔΨ were also normalized by dividing their magnitude by ECS_{ST}. The time constant of the first-order ECS relaxation (τ_{ECS}) is inversely proportional to the proton conductivity (g_H^+) of the thylakoid membrane through the ATP synthase (Sacksteder and Kramer 2000; Cruz et al. 2005). As a result, g_H^+ was estimated as the inverse of the decay time constant [$1/\tau_{ECS}$]. The relative light-driven proton flux was calculated as $v_H^+ = ECS_t/ECS_{ST} \times g_H^+$.

Statistical analysis

The results were displayed as mean values of at least six independent experiments. The data were subjected to analysis of variance (ANOVA) using the SPSS 16.0 statistical software. One-Way ANOVA test was used at $\alpha = 0.05$ significance level to determine whether significant differences existed between mature and immature leaves.

Results

Phenotypic comparison of photosynthetic traits

For the mature and immature leaves, the rates of CO_2 assimilation (A_n) at 1500 µmol photons m^{-2} s^{-1} and 400 µmol mol^{-1} CO_2 concentration were 9.1 and 3.1 µmol m^{-2} s^{-1}, respectively (Fig. 1a), indicating the significantly lower photosynthetic capacity in the immature leaves. The maximum quantum yields of PSII after dark adaptation (F_v/F_m) were 0.83 and 0.77 in the studied mature and immature leaves, respectively (Fig. 1b), suggesting that the development of PSII function was not accomplished in the immature leaves. However, the large difference in A_n between the immature and mature leaves was not caused by the change in F_v/F_m, suggesting that PSII activity is not the rate-limiting factor for CO_2 assimilation in the immature leaves.

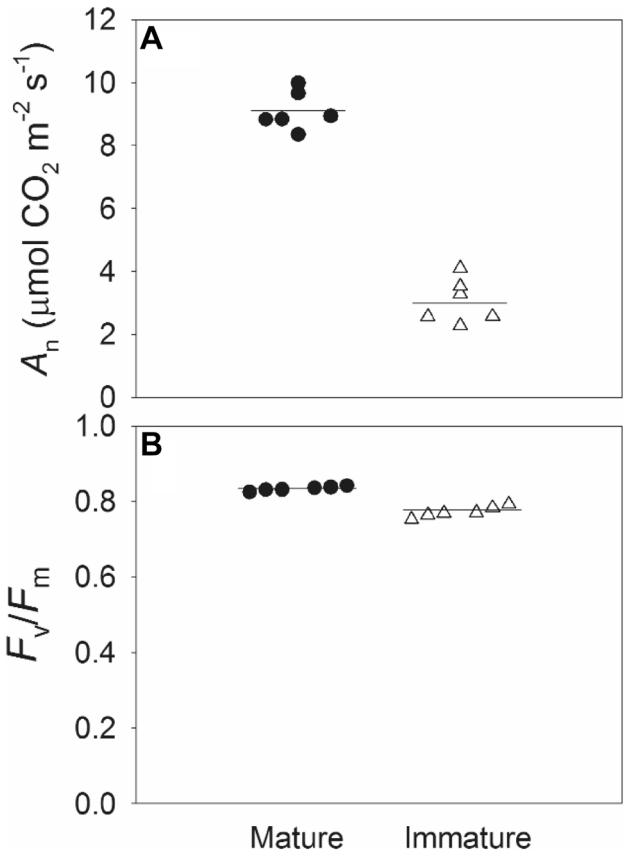

Fig. 1 Phenotypic comparison of photosynthetic traits of mature and immature leaves. **a** CO_2 assimilation rate at 1500 µmol photons m^{-2} s^{-1} and 400 µmol mol^{-1} CO_2 concentration, **b** the maximum quantum yield of PSII (F_v/F_m)

Light intensity dependence of PSI and PSII parameters

Light response curves indicated that the effective quantum yield of PSII [Y(II)] in the immature leaves were significantly lower than that in mature leaves, irrespective of light intensity (Fig. 2a), suggesting that immature leaves exhibit lower ability to utilize the product of linear electron flow. The fraction of open PSII reaction centers, qL, decreased gradually with increasing light intensity (Fig. 2b). The immature leaves showed significantly lower qL than the mature leaves, excluding at 54 µmol photons m^{-2} s^{-1} (Fig. 2b). Non-photochemical quenching in PSII, NPQ, increased in different ways with increasing light intensity between mature and immature leaves (Fig. 2c). In the immature leaves, NPQ rapidly increased and reached the maximum value at 325 µmol photons m^{-2} s^{-1} (Fig. 2c). By comparison, NPQ reached the maximum value at 606 µmol photons m^{-2} s^{-1} in the mature leaves (Fig. 2c). Under light intensities below 325 µmol photons m^{-2} s^{-1}, immature leaves showed significantly higher NPQ than the mature leaves (Fig. 2c). The mature and immature leaves showed similar NPQ capacity under saturating light intensities.

The quantum yield of PSI photochemistry, Y(I), decreased gradually with increasing light intensity in both mature and immature leaves (Fig. 3a), in accordance with previous results (Huang et al. 2017a). Values for Y(I) were significantly higher in mature leaves when compared with immature leaves, excluding at 54 µmol photons m^{-2} s^{-1} (Fig. 3a). With increasing light intensity, the quantum yield of PSI non-photochemical energy dissipation due to the donor-side limitation, Y(ND), gradually increased in both mature and immature leaves (Fig. 3b), which is in accordance with previous results reported in wild-type plants (Munekage et al. 2002, 2004; Kono et al. 2014; Huang et al. 2017a). Interestingly, the values for Y(ND) were significantly higher in immature leaves compared to mature leaves, irrespective of light intensity (Fig. 3b). The quantum yield of PSI non-photochemical energy dissipation due to the acceptor-side limitation, Y(NA) responded in different ways to various light intensities. In mature leaves, Y(NA) gradually decreased with increasing light intensity (Fig. 3c). By comparison, Y(NA) was lower than 0.1 under all light intensities in the immature leaves (Fig. 3c), indicating the prevention of over-reduction of PSI acceptor side in immature leaves.

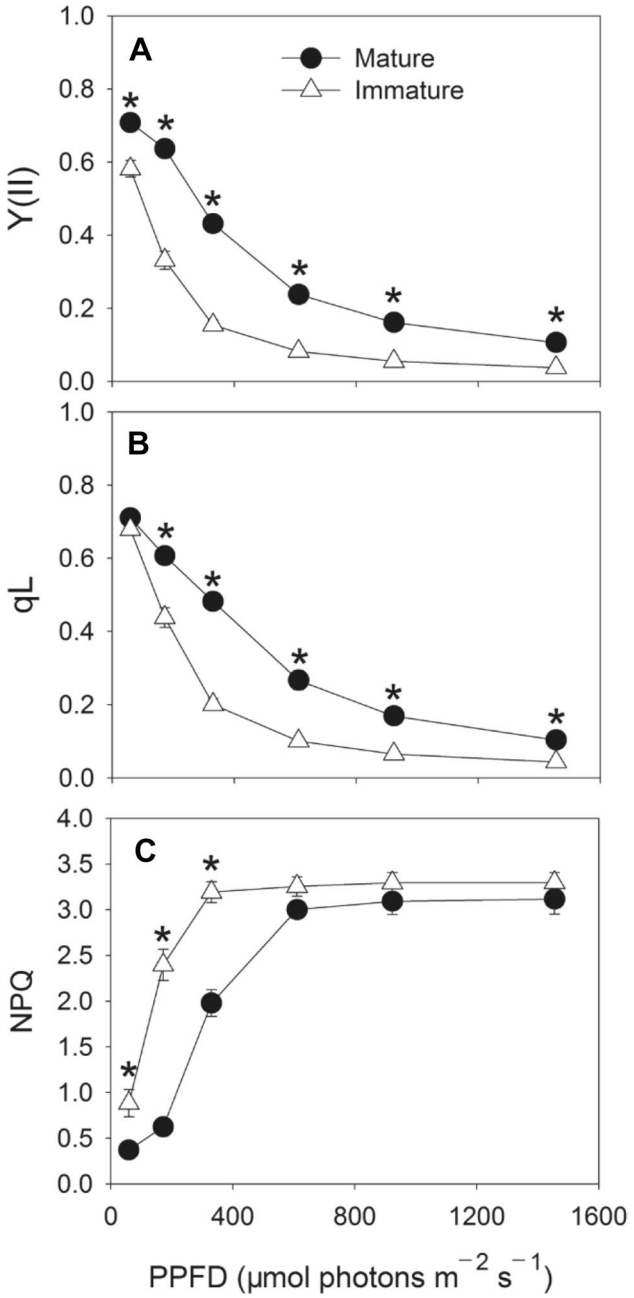

Fig. 2 Light intensity dependence of Y(II) (**a**), qL (**b**), and NPQ (**c**) for mature and immature leaves. Y(II), effective quantum yield of PSII photochemistry; qL, the fraction of open PSII reaction centers based on the "lake model" of PSII antenna pigment organization; NPQ, non-photochemical quenching in PSII. Values are means ± SE ($n = 6$). Asterisks indicate significant differences between mature and immature leaves

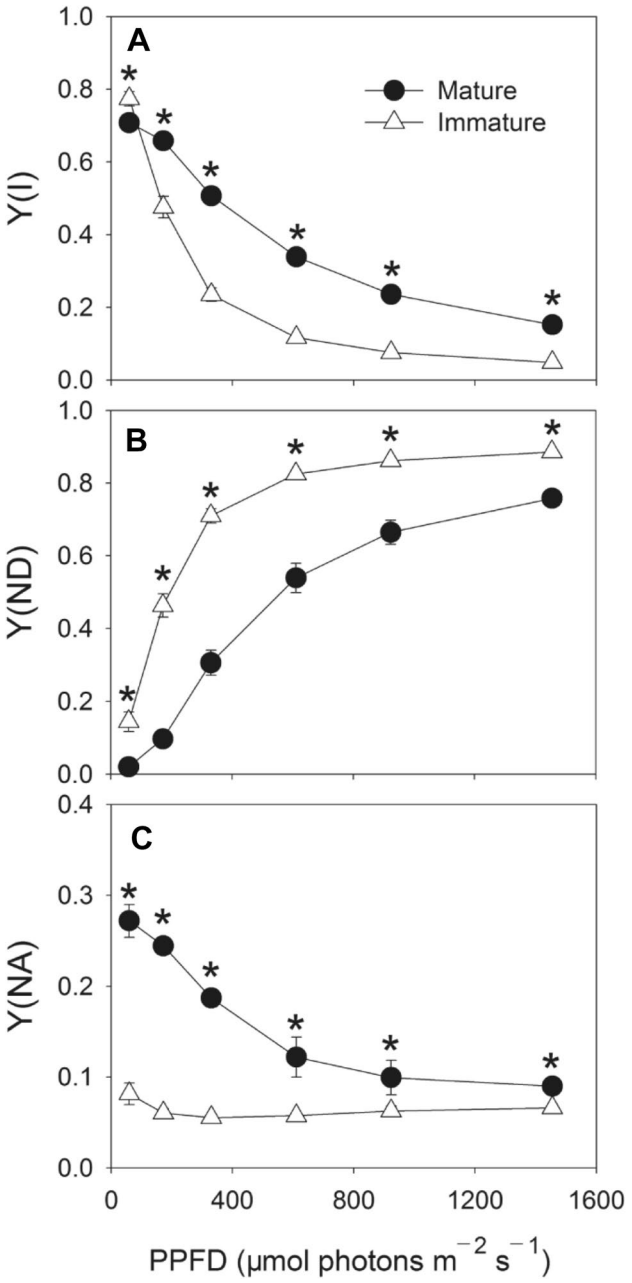

Fig. 3 Light intensity dependence of Y(I) (**a**), Y(ND) (**b**), and Y(NA) (**c**) for mature and immature leaves. Y(I), quantum yield of PSI photochemical quenching; Y(ND), the quantum yield of PSI non-photochemical energy dissipation due to the donor-side limitation; Y(NA), the quantum yield of PSI non-photochemical energy dissipation due to the acceptor-side limitation. Values are means ± SE ($n = 6$). Asterisks indicate significant differences between mature and immature leaves

Under all light intensity, Y(NA) values were significantly lower in the immature leaves.

The data of Y(II) mean that the immature leaves showed lower LEF activity than the mature leaves. In addition, we compared the CEF activity estimated from the relationship between Y(I) and Y(II) (Fig. 4). The immature leaves showed nearly uniformly high ratios of Y(I)–Y(II) across nearly all light intensities, while in mature leaves the ratio is low at lower light intensities but increases to match the ratios observed in immature leaves at higher light intensities.

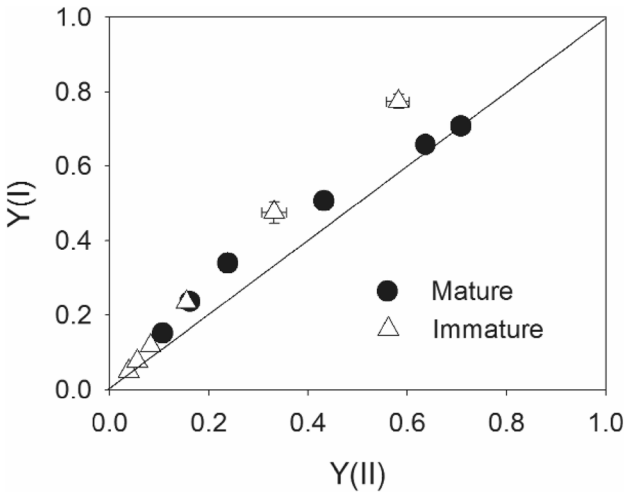

Fig. 4 Comparison of cyclic electron flow around PSI for mature and immature. The relationship between Y(I) and Y(II). Values are means ± SE ($n = 6$)

These results indicated that the activation of CEF at low light intensities was higher in the immature leaves (Livingston et al. 2010). In mature leaves, CEF was significantly induced at high light.

Proton motive force and thylakoid proton conductivity

To understand the light-dependent formation of proton motive force, the responses of $DIRK_{ECS}$ kinetics to various light intensities are shown in Fig. 5. The total pmf was found to be significantly higher in the immature leaves than the mature leaves, excluding at 1450 µmol photons m^{-2} s^{-1} (Fig. 5a). The proton gradient (ΔpH) component of pmf gradually increased with increasing light intensity (Fig. 5b). Furthermore, the immature leaves showed significantly higher ΔpH than the mature leaves under light intensities below 1450 µmol photons m^{-2} s^{-1} (Fig. 5b). Meanwhile, the immature and mature leaves showed similar values of the electrochemical gradient (ΔΨ) component of pmf, excluding at a low light of 54 µmol photons m^{-2} s^{-1} (Fig. 5c).

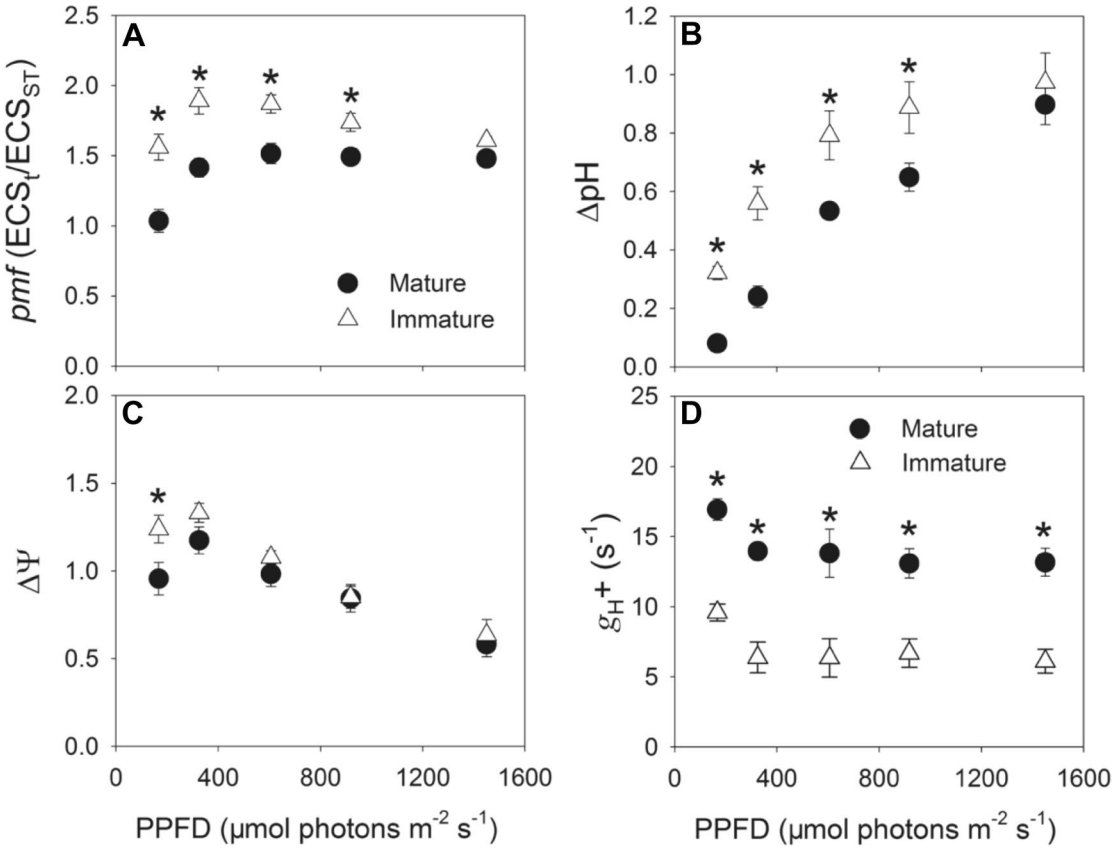

Fig. 5 Parameters derived from the dark-interval relaxation kinetic of ECS ($DIRK_{ECS}$) recorded after 5 min illumination at different light intensities for mature and immature leaves. Before this analysis, leaves were illuminated at 606 µmol photons m^{-2} s^{-1} for 15 min to activate the electron sink in photosynthesis. **a** Total proton motive force (pmf) estimated from the amplitude of ECS decay; **b** The proton gradient (ΔpH) component of pmf; **c** The electrochemical gradient (ΔΨ) component of pmf; **d** The thylakoid proton conductivity (g_H^+). Values are means ± SE ($n = 6$). Asterisks indicate significant differences between mature and immature leaves

Importantly, the immature leaves showed much lower values of thylakoid proton conductance (g_H^+) than the mature leaves, irrespective of light intensity (Fig. 5d). Because the conductivity of thylakoid proton efflux is attributable to the activity of chloroplast ATP synthase (Kanazawa and Kramer 2002; Avenson et al. 2005; Kohzuma et al. 2009; Kanazawa et al. 2017; Takagi et al. 2017), the lower g_H^+ values in the immature leaves are assumed to be partly caused by the lower activity of chloroplast ATP synthase (Kuhlgert et al. 2016). For a given leaf, the activity of chloroplastic ATP synthase is thought to be regulated by Pi (Kanazawa and Kramer 2002; Takizawa et al. 2008; Kohzuma et al. 2009; Karlsson et al. 2015). Under high light, ATP consumption is slow relative to ATP synthesis, and the availability of Pi decreases. Consistently, g_H^+ decreased under high light in both mature and immature leaves.

To further analyze photosynthetic regulation in immature leaves, we plotted data from Figs. 2c, 3b, and 5b to produce Fig. 6. Figure 6a shows that the response of NPQ to ΔpH in the immature leaves is similar to the mature leaves. The maximum NPQ can be reached at a value of ΔpH being 0.6 (Fig. 6a), indicating that the ΔpH required for the protonation of PsbS and violaxanthin de-epoxidase was lower than the maximum ΔpH leaves have. However, as shown in Fig. 6b, Y(ND) gradually increased with the increasing ΔpH. Therefore, the increased ΔpH under high light would be more important for adjusting PSI redox state than for NPQ induction.

The chlorophyll fluorescence obtained using red measuring light (Dual PAM) will be predominantly from the upper parts of the leaf whereas Y(I) will be from the whole leaf as it is an absorption measurement. This can lead to overestimation of CEF (Kou et al. 2013). In order to further analyze the contribution of CEF in proton flux, we examine the relationship between relative light-driven proton flux (v_H^+) and LEF (Fig. 7). v_H^+ reflects the total proton flux generated by both LEF and CEF, while the LEF only indicates the electron transfer from PSII. At a low light of 172 µmol photons m^{-2} s^{-1}, values for LEF were 46 and 24 µmol electrons m^{-2} s^{-1} in the mature and immature leaves, respectively (Fig. 7a). Meanwhile, the v_H^+ in the immature leaves was complemented to the mature leaves level (Fig. 7b). As a result, at this low light, the proton flux from CEF was much higher in the immature leaves.

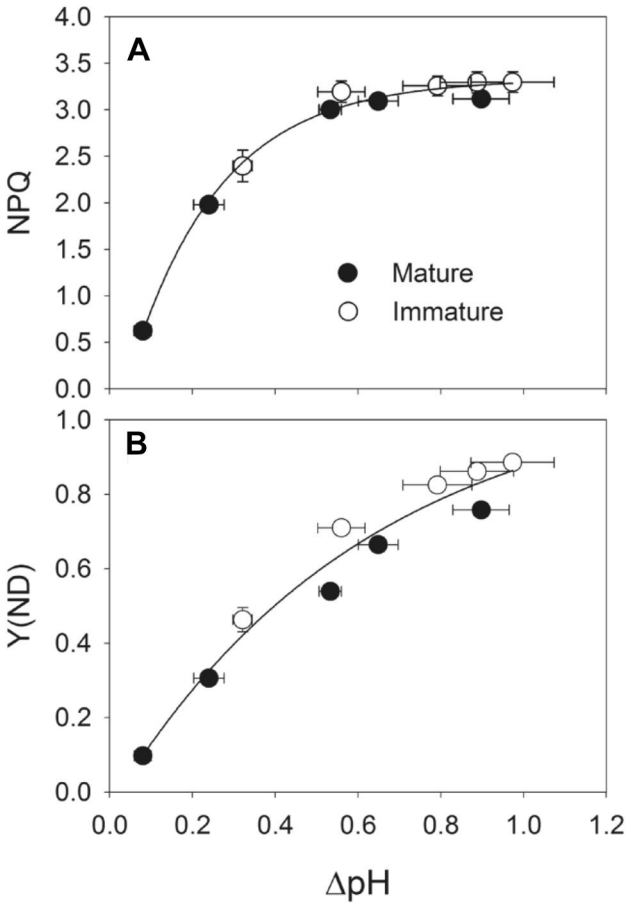

Fig. 6 Analysis of photosynthetic regulation in mature and immature leaves. **a** Relationship between ΔpH and NPQ; **b** Relationship between ΔpH and Y(ND). Light response data given in Figs. 2c, 3b, and 5b were used

Discussion

Previous studies indicated that both biochemical and structural limitations restricted the light use efficiency in developing leaves (Niinemets et al. 2012). Consistently, the immature leaves displayed much lower photosynthetic capacity than the mature leaves (Fig. 1a). For a given leaf, inhibition of CO_2 assimilation can accelerate the production of ROS (Takahashi and Murata 2005; Murata et al. 2007). As a result, the relatively low A_n can increase the risk of ROS production in the immature leaves. It has been documented that ROS can aggravate photoinhibition of PSII through two different mechanisms: one is increasing the rate of photodamage by oxidative damage (Krieger-Liszkay et al. 2011), and the other one is the inhibition of photodamaged PSII repair at the step of protein synthesis (Nishiyama et al. 2001, 2006, 2011). Furthermore, superoxide and singlet oxygen generated within the thylakoid membranes both cause photoinhibition of PSI when electron carriers in PSI become highly reduced (Takagi et al. 2016, 2017). As a result, immature leaves should maintain the photosynthetic electron chain in a more oxidized state and diminish the production of ROS, which is crucial for photoprotection for PSI and PSII.

Proton motive force plays a central role in photosynthesis and regulation of photosynthetic electron flow (Kramer et al. 2003, 2004; Shikanai 2014, 2016; Yamori and Shikanai 2016; Shikanai and Yamamoto 2017). Once the

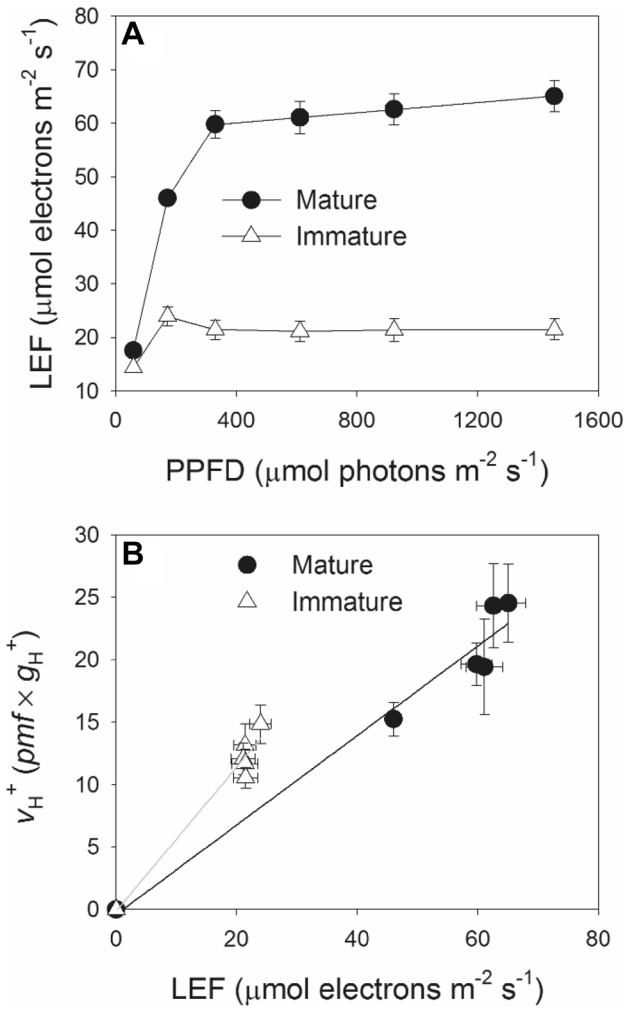

Fig. 7 Light intensity dependence of linear electron flow (LEF) (**a**) and the relationship between relative light-driven proton flux (v_H^+) and LEF (**b**). Values are means ± SE ($n = 6$)

generation of ΔpH was impaired under excess light, P700 would be highly reduced, leading to the production of ROS within PSI and thus causing PSI photoinhibition (Takagi et al. 2016, 2017; Kanazawa et al. 2017). Proton motive force is composed of ΔΨ and ΔpH, and both ΔΨ and ΔpH contribute to the ATP synthesis through the chloroplastic ATP synthase. In higher plants, photoprotection and photosynthetic electron flow are mainly regulated by ΔpH. The generation of lumen acidification is dependent on two factors: (1) the accumulation of protons in the lumen from water-splitting activity of PSII and from the electron transfer via Cyt b_6/f; and (2) the rate of proton efflux from lumen to stroma (i.e., activity of ATP synthase in releasing the ΔpH). The Cyt b_6/f complex couples the electron transfer (LEF and CEF) to proton translocation. This not only enhances the formation of ΔpH, but also allows the control of electron transfer from PSII to PSI according to the ΔpH (Suorsa et al. 2012; Tikkanen and Aro 2014; Shikanai 2014, 2016). At lower light intensities, an apparent increase in CEF and lower g_H^+ contributed to a larger ΔpH in the immature leaves compared to mature leaves. Under high light, the immature leaves displayed significantly lower values of Y(I) and Y(II) (Figs. 2a, 3a), suggesting the lower photosynthetic electron flow compared to mature leaves. Meanwhile, the immature leaves showed higher levels of pmf and ΔpH than the mature leaves (Fig. 5a, b). Therefore, we cannot explain the cause of the higher pmf and ΔpH in the immature leaves by the difference in proton influx activity in dependence on the photosynthetic electron flow. In contrast, the immature leaves showed much lower g_H^+ than the mature leaves under high light (Fig. 5d). These results indicated that the higher pmf and ΔpH under high light in the immature leaves are mainly caused by the lower rate of proton efflux from lumen to stroma. Under high light, the deficiency of proton influx activity in immature leaves is compensated by the lower activity of ATP synthase during photosynthesis. Based on these findings, we propose that the relatively lower activity of ATP synthase is an important strategy used by immature leaves to regulate pmf and ΔpH.

Many previous studies have confirmed the critical role of lumen acidification in photoprotection for PSII. The lumen acidification helps to alleviate PSII photoinhibition by at least two different mechanisms: one is linked to thermal energy dissipation and prevents the inhibition of the repair of photodamaged PSII at the step of protein synthesis, the other one is independent of thermal energy dissipation and suppresses the rate of PSII photodamage (Takahashi et al. 2009; Huang et al. 2016a). The PsbS protein in the antenna system senses the lumen acidification, allowing safe dissipation of the excess excitation energy as heat (Li et al. 2002). Furthermore, the energy transfer efficiency from LCHII to the photosynthetic electron chain is reduced by increased lumen acidification, which then decreases ROS production and favors the repair of PSII (Takahashi et al. 2009). Interestingly, the immature leaves had an efficient system of thermal energy dissipation (as indicated by the high levels of NPQ, Fig. 2c). The rate of linear electron flow in the immature leaves was saturated at much lower light when compared with mature leaves. Concomitantly, NPQ was saturated in immature leaves at lower light. The relationship between ΔpH and NPQ indicated that the higher NPQ in immature leaves at low light was caused by the higher ΔpH, which activates the xanthophyll cycle (Horton et al. 2000). As a result, the immature leaves probably showed higher de-epoxidation at low light. On the other hand, lumen acidification 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; Takahashi et al. 2009). Therefore, the high levels

of NPQ activation and lumen acidification both protect PSII against photoinhibition in immature leaves.

As indicated in wild-type and *pgr5* plants of *Arabidopsis thaliana*, ΔpH plays a crucial role in the induction of NPQ (Munekage et al. 2002, 2004; Yamamoto et al. 2016). However, the relationship between NPQ activation and ΔpH is not well known. Under drought stress, the significant increase in ΔpH under high light did not lead to a significant increase in NPQ (Zivcak et al. 2014). Our results indicated that the full activation of NPQ can be reached at a relatively lower ΔpH than the maximum ΔpH leaves have (Fig. 6a). Furthermore, the response of NPQ to ΔpH in immature leaves was similar to mature leaves (Fig. 6a). Therefore, in addition to NPQ activation, the enhanced ΔpH under high light has other more important physiological functions. When NPQ was saturated, the increase in ΔpH at higher light intensities contributed to the further increase in Y(ND), suggesting the central role of ΔpH in the redox adjustment of P700 in PSI. As a result, this increase in ΔpH would be more important for oxidizing PSI than for inducing NPQ, which is consistent with previous studies (Tikkanen et al. 2015; Takagi et al. 2017).

Although PSII photoinhibition is an inevitable process under high light, photodamaged PSII complex can recover rapidly in several hours (He and Chow 2003; Aro et al. 2005; Oguchi et al. 2008; Allakhverdiev 2011). In contrast, recovery of PSI from photoinhibition is a slow process (Kudoh and Sonoike 2002; Zhang and Scheller 2004; Zivcak et al. 2015), and thus, PSI photoinhibition has more severe consequences than that of PSII. Typically, PSI photoinhibition is caused by the accumulation of reduced electron carriers (Munekage et al. 2002; Allahverdiyeva et al. 2005; Suorsa et al. 2012; Kono et al. 2014; Tikkanen et al. 2014; Takagi et al. 2016, 2017), though the occurrence of PSI photoinhibition has been shown to be independent of PSI redox state in some shade-establishing plants such as *Psychotria rubra* and *Psychotria henryi* (Huang et al. 2015, 2016b, 2017b). In the *pgr5*-plants, the deficiency of proton influx activity plus the higher proton efflux to stroma lead to the loss of lumen acidification (Avenson et al. 2005; Wang et al. 2015), resulting in excess electron flow to PSI and severe PSI photoinhibition under high or fluctuating light intensities (Suorsa et al. 2012; Tikkanen et al. 2014; Yamori et al. 2016). In *cfq* and *hope2* mutants of *Arabidopsis thaliana*, the higher proton efflux activity from the lumenal to stromal side of thylakoid membranes suppressed the formation of ΔpH during photosynthesis, resulting in a more reduced state of PSI, thus causing PSI photoinhibition under high light or fluctuating light (Kanazawa et al. 2017; Takagi et al. 2017). In contrast, ATP synthase repression can restrict photosynthetic electron transport by means of over-acidification of the thylakoid lumen in tobacco (Rott et al. 2011). Here, we observed that the P700 oxidation ratio, namely Y(ND), was significantly higher in the immature leaves than the mature leaves (Fig. 3b). As long as high Y(ND) is maintained under high light, immature leaves can escape ROS production within the thylakoid membranes, thus preventing PSI photoinhibition. Importantly, under such conditions, g_H^+ was depressed and ΔpH was enhanced in the immature leaves (Fig. 5). These findings indicate that the immature leaves lower the proton efflux activity to contribute the oxidation of P700 through buildup of ΔpH, which is essential for avoidance of PSI photoinhibition during photosynthesis under excess light.

In summary, the present study examined the regulation of proton motive force in immature leaves. Immature leaves showed much lower photosynthetic electron flow (including LEF and CEF) under high light. Moreover, we found that under high light the immature leaves had a lower g_H^+ and higher pmf than the mature leaves. These results indicate that immature leaves depress the proton efflux activity in ATP synthase to compensate for the deficiency of proton influx activity in dependence on photosynthetic electron flow, leading to the buildup of a sufficient pmf under excess light. This response would stimulate the acidification of thylakoid lumen and trigger both the activation of NPQ and the photosynthetic control of electron transfer at Cyt b_6/f. Consequently, photosynthetic apparatus can be largely protected under high light, especially for PSI activity. Taking together, this study highlights the important role of ATP synthase in regulation of proton motive force and photoprotection in immature leaves.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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