



# Photosynthetic regulation under fluctuating light in young and mature leaves of the CAM plant *Bryophyllum pinnatum*



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## ARTICLE INFO

### Keywords:

Photoprotection  
Photosystem I  
Proton gradient  
Redox state of P700  
Water-water cycle  
Leaf development

## ABSTRACT

Photosystem I (PSI) is the potential target of photodamage under fluctuating light in angiosperms. However, the response of PSI to fluctuating light in young leaves has not yet been clarified. Furthermore, the photosynthetic regulation under fluctuating light in crassulacean acid metabolism (CAM) plants is little known. In this study, we measured PSI redox state and the electrochromic shift signal in the mature and young leaves of a CAM species *Bryophyllum pinnatum*. The mature leaves showed stronger capacity for photo-reduction of O<sub>2</sub> mediated by the alternative electron flow (probably the water-water cycle) when compared with the young leaves. After an increase in light intensity, both the mature and young leaves showed insufficient proton gradient ( $\Delta pH$ ) across the thylakoid membranes within the first seconds. Meanwhile, PSI was highly oxidized in the mature leaves but was in a more reduced state in the young leaves. Furthermore, young leaves were more susceptible to PSI photoinhibition under fluctuating light. Therefore, in the mature leaves, the alternative electron flow significantly optimized the PSI redox state under fluctuating light at relatively low  $\Delta pH$ . By comparison, in the young leaves, PSI redox state was largely determined by the buildup of  $\Delta pH$ . Therefore, the major photo-protective mechanism responsible for safeguarding PSI under fluctuating light can be influenced by leaf developmental stages.

## 1. Introduction

Plants absorb light energy to drive photosynthetic electron transport that converts light energy into the chemical energy. In linear electron flow, electrons from water are transported to the cytochrome (Cyt) *b<sub>6</sub>/f* complex and photosystem I (PSI), and ultimately to NADP<sup>+</sup>, to produce NADPH, which is coupled with the formation of a proton gradient ( $\Delta pH$ ) across the thylakoid membranes. By comparison, cyclic electron transport (CET) around PSI generates a  $\Delta pH$  without producing NADPH. The cooperation of linear and cyclic electron transport balances the production of ATP and NADPH, thus optimizing the ATP/NADPH ratio required by the Calvin cycle, photorespiration and other primary metabolism [1–6].

Natural environmental conditions are highly variable, and a sudden increase in irradiation can drastically increase the absorbed light. Under such conditions, the electrons transported to PSII to PSI cannot be effectively consumed by carbon fixation and other primary metabolism, generating a dangerous imbalance between electron flow to PSI and outflow of electrons to NADP<sup>+</sup> [7,8]. This over-reduction of PSI electron carriers induces the ROS production within PSI, thus causing

photodamage to PSI. In fact, PSI photoinhibition impairs the capacity of CO<sub>2</sub> assimilation and plant growth [9–16]. Furthermore, once the photoinhibition occurs, PSI recovers very slowly, which needs several days [17,18]. Therefore, PSI photoinhibition should be avoided in plants to enable normal growth under natural fluctuating light.

Photosynthetic organisms evolved several mechanisms to regulate the redox state of PSI upon an increase in light intensity. In non-flowering plants, the flavodiiron (Flv) proteins rapidly accept electrons from PSI to O<sub>2</sub>, and thus avoid over-reduction of electron transport chain [8,19]. As a result, Flvs are the main player enabling them growth under fluctuating light [20,21]. In contrast, *Flv* genes are absent in angiosperms [19], and CET has been previously regarded as the main player for sustaining photosynthesis and growth under fluctuating light [6,11,22–24]. Impairment of CET induces severe photoinhibition of PSI under fluctuating light [11,13,14,25]. As a consequence, the *pgr5* mutant dies at the seedling stage when grown under fluctuating light [11,25]. However, when wild-type and *pgr5* plants were first grown under constant light until development of the mature rosettes, *pgr5* plants survived for weeks after transfer to fluctuating light [11]. Furthermore, the PSI content in mature leaves remains rather stable, but

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<https://doi.org/10.1016/j.bbabio.2019.04.006>

Received 23 February 2018; Received in revised form 20 December 2018; Accepted 6 January 2019

Available online 25 April 2019

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varies more in young leaves [26]. These reports suggest that PSI is more susceptible to fluctuating light in the young leaves compared with mature leaves [7]. However, the underlying mechanisms remain to be clarified.

Young leaves have lower PSI activity and photosynthetic capacity than mature leaves, leading to more excess excitation energy under high light in young leaves [27–29]. The excess excitation energy can induce the production of reactive oxygen species (ROS) within thylakoid membranes or in the chloroplast stroma, thus causing photo-inhibition of PSI [30–34]. Our recent studies indicated that young leaves displayed high levels of non-photochemical quenching (NPQ) and P700 oxidation ratio during steady state photosynthesis under high light [27,29], owing to the higher value of  $\Delta\text{pH}$  modulated by the low thylakoid proton conductivity [28,29]. However, it is unclear whether the young leaves can build up an enough  $\Delta\text{pH}$  upon a sudden increase in light intensity, which complicates our understanding of photosynthetic regulation in young leaves.

In *pgr5* mutant of *Arabidopsis thaliana*, the formation of  $\Delta\text{pH}$  under high light was depressed [22,23], owing to the reduction of proton influx activity and enhanced thylakoid proton conductivity [35–37]. As a result, *pgr5* mutant lacks  $\Delta\text{pH}$ -dependent down-regulation of plastoquinol oxidation at the cytochrome *b<sub>6</sub>/f* complex [11,38], resulting in excess electron flow from PSII to PSI and thus photoinhibition of PSI under fluctuating light and high light [11,25,39]. Thus, it is conceivable that  $\Delta\text{pH}$  plays a key role for regulating PSI redox state and thus protecting PSI under fluctuating light in angiosperms [36,40]. However, for the first seconds after an increase in illumination, PSI is in a highly reduced state in the wild-type plants of *Arabidopsis thaliana* [41,42], rice [43] and *Bletilla striata* [44,45]. As a result, we hypothesize that the lumen acidification of thylakoid has a slower kinetics compared with electron flow, and thus the  $\Delta\text{pH}$ -dependent photosynthetic control has its deficiency under fluctuating light.

At present, many studies have investigated the photosynthetic regulation under fluctuating light in  $C_3$  angiosperms [13,41,43,46]. However, any direct evidence for how quickly crassulacean acid metabolism (CAM) leaves deal with rapid changes in light intensity is completely missing. Because CAM plants close stomata in the daytime, their photosynthetic regulation under fluctuating light is independent of stomatal behavior, which is opposite to the  $C_3$  plants. In this study, we studied the photosynthetic regulation under fluctuating light in the mature and young leaves of *B. pinnatum*. The aims of the study are: (1) to examine whether young leaves are more susceptible to PSI photoinhibition under fluctuating light than mature ones; (2) to assess the physiological determinants for such strong PSI photoinhibition in the young leaves; (3) to explore the photosynthetic regulation under fluctuating light in CAM plants?

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

*Bryophyllum pinnatum* (Linnaeus f.) Oken is a CAM plant native to Africa and has been cultivated widely in China. In the present study, plants of *B. pinnatum* were grown in a greenhouse with high relative air humidity (60%–70%) and 40% of full sunlight. These plants of *B. pinnatum* were cultivated without water or nutrition stresses. Mature fully expanded leaves flushed two months ago and young leaves flushed within three weeks were chosen for photosynthetic measurements. The average leaf area of the mature leaves was approximately four times the young leaves (data not shown). When measured at 25 °C in the daytime, the maximum rates of PSII electron flow in the mature and immature leaves were about 70 and 38  $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$  (data not shown).

### 2.2. Chlorophyll fluorescence and P700 measurements

PSI and PSII parameters were recorded simultaneously at 25 °C

using a Dual-PAM 100 measuring system (Heinz Walz, Effeltrich, Germany). After dark adaptation for 30 min, a saturating pulse was applied to measure the maximum fluorescence and the maximum change in P700, and then leaves were illuminated at a saturating light of 1455  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 10 min to activate photosynthetic electron sinks, followed by illumination at 59  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 5 min. Afterward, the PSI and PSII parameters were measured under fluctuating light alternating between 59 and 1455  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

The PSI photosynthetic parameters were measured according to the method described in [47]. The P700<sup>+</sup> signals (*P*) could vary between a minimum (P700 fully reduced) and a maximum level (P700 fully oxidized). The maximum,  $P_m$ , was determined by applying a saturation pulse (300 ms and 20,000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) after pre-illumination with far-red light for 10 s.  $P_m'$  was similarly obtained, except that actinic light was used instead of far-red light. Calculations of PSI parameters included the quantum yield of PSI photochemistry,  $Y(I) = (P_m' - P) / P_m$ ; the quantum yield of PSI non-photochemical energy dissipation due to donor side limitation,  $Y(\text{ND}) = P / P_m$ ; the quantum yield of non-photochemical energy dissipation due to acceptor side limitation,  $Y(\text{NA}) = (P_m - P_m') / P_m$ . The P700 redox state during dark-to-light transition was also measured using a Dual-PAM 100. After dark-adaptation for at least 60 min, the P700 redox changes of P700 were recorded during 10 s illumination at 1455  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

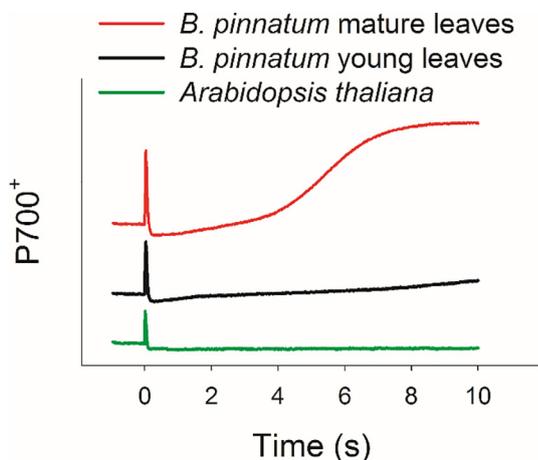
The effective quantum yield of PSII was calculated as  $Y(\text{II}) = (F_m' - F_s) / F_m'$  [48]. The non-photochemical quenching in PSII was  $\text{NPQ} = (F_m - F_m') / F_m'$ .  $F_m$  and  $F_m'$  represent the maximum fluorescence after dark-adaptation and light-adaptation, respectively.  $F_s$  is the light-adapted steady-state fluorescence.

### 2.3. Electrochromic shift analysis

The Electrochromic shift (ECS) signal was monitored as the change in absorbance at 515 nm [35], using a Dual PAM-100 equipped with a P515/535 emitter-detector module (Heinz Walz). After dark-adaptation for at least 30 min, the 515-nm absorbance change induced by a single turnover flash ( $\text{ECS}_{\text{ST}}$ ) was measured. Subsequently, ECS signals after the transition from dark to 1455  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 20, 120 and 600 s were measured with an interval of 10 min dark adaptation. Afterwards, the same leaves were illumination at 59  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 5 min, and then the actinic light was changed to 1455  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and the ECS signal was measured after this light transition for 10 s. Subsequently, the ECS signal after the transition from 59 to 1455  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 120 s was measured following a 5 min adaptation at 59  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . We analyzed ECS dark interval relaxation kinetics ( $\text{DIRK}_{\text{ECS}}$ ) as described by [49,50]. The difference in proton motive force (*pmf*) between light and dark,  $\text{ECS}_t$ , was estimated from the total amplitude of the rapid decay of the ECS signal during the dark pulse. All  $\text{ECS}_t$  levels were normalized against the magnitude of  $\text{ECS}_{\text{ST}}$ . This normalization accounted for variations in leaf thickness and chloroplast density among the leaf samples [28,35,50,51]. The slow relaxation of the ECS signal was measured to calculate the  $\Delta\text{pH}$  and membrane potential ( $\Delta\Psi$ ) [52,53]. The proton conductivity of the thylakoid membrane through ATP synthase ( $\text{gH}^+$ ) was also calculated [53].

### 2.4. Photoinhibitory treatments

In the present study, light from a 635 nm light-emitting diode (LED) equipped in a Dual-PAM-100 was used as actinic light for photoinhibitory treatments. After dark adaptation for 30 min, the initial value of  $P_m$  was measured. Subsequently, leaves were exposed to fluctuating light alternating between 59 and 1455  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  every 20 s. Following treatments for 800, 1600 and 2400 s,  $P_m$  was determined after dark adaptation for 3 min. It should be noted that 1455  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  is a saturating light for all leaves of the



**Fig. 1.** Representative redox kinetics of P700 upon the illumination of dark-adapted mature and young leaves of *Bryophyllum pinnatum* as compared with *Arabidopsis thaliana*. After dark adaptation for at least 60 min, the kinetics of redox changes were measured *in vivo* upon exposure to actinic light ( $1455 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ).

CAM plant *B. pinnatum*.

## 2.5. Statistical analysis

The results are displayed as the mean values of five independent experiments. One-way ANOVA was used at the  $\alpha = 0.05$  significance level to determine whether significant differences existed between different treatments.

## 3. Results

### 3.1. P700 redox kinetics upon abrupt illumination of dark-adapted leaves

We first determined the P700 redox kinetics upon the illumination of dark-adapted leaves to actinic light ( $1455 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), to attempt to examine the fast regulation of PSI redox state in the mature and young leaves of *B. pinnatum* (Fig. 1). In the mature leaves, actinic light induced the initial peak of P700 oxidation, which was followed by its reduction and the fast re-oxidation of P700. These processes were accomplished in 8 s. By comparison, the re-oxidation of P700 was not observed in the young leaves, as was in experiments with wild-type *Arabidopsis thaliana* (Fig. 1). Furthermore, in the mature leaves, the fast re-oxidation of P700 was clearly missing under anaerobic condition (Fig. S1). As a result, the fast re-oxidation of P700 in the mature leaves indicated the photo-reduction of  $\text{O}_2$  mediated by alternative electron flow in PSI. Consequently, the mature leaves had stronger capacity for the alternative electron flow from PSI to  $\text{O}_2$  compared with the young leaves.

### 3.2. Changes in PSI and PSII parameters after onset of light

Next, we examined the PSI and PSII parameters during photosynthetic induction. When dark-acclimated leaves were exposed to a saturating light of  $1455 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 20 s, the mature leaves showed a stronger light-dependent PSI oxidation [Y(ND); Fig. 2A], whereas the young leaves showed a stronger PSI acceptor side limitation [Y(NA); Fig. 2B]. The large differences in Y(ND) and Y(NA) between the mature and young leaves were only present in the first seconds after the light was switched on and progressively decreased during illumination (Fig. 2). These results indicated that the mature leaves have a stronger capacity to rapidly oxidize P700 for the first seconds upon dark-to-light transition as compared with young leaves. The non-photochemical quenching (NPQ) in PSII was gradually

activated during photosynthetic induction (Fig. 2C), suggesting the formation of  $\Delta\text{pH}$  in both types of leaves. Furthermore, under steady state photosynthesis at this high light, young leaves showed stronger NPQ than mature ones (Fig. 2C).

After the saturating light was switched on, quantum yield of PSI photochemistry, Y(I), gradually increased in the mature leaves, then reaching a steady state (Fig. 2D). By comparison, after the light was switched on for 20 s, Y(I) was first stimulated to a much higher value in the young leaves. Afterward, Y(I) rapidly decreased during the 20 to 60 s, and then gradually increased and reached a steady state (Fig. 2D). Upon transition from dark to light, quantum yield of PSII photochemistry, Y(II), gradually increased and then reached a steady state in both mature and young leaves (Fig. 2E). Under steady state condition, the mature leaves showed much higher values of Y(I) and Y(II) than the young leaves, suggesting the higher photosynthetic electron flow in the mature leaves. However, the young leaves displayed much higher Y(I)/Y(II) ratios for the first seconds after the actinic light was switched on (Fig. 2F). After reaching the steady state, there was no significant difference in Y(I)/Y(II) ratio between the mature and young leaves (Fig. 2F). Because Y(I)/Y(II) ratio is an indicator of the activation of CET around PSI in higher plants [2,3,54,55], these results suggested that the young leaves showed an increased cyclic electron flow for the first seconds during dark-to-light transition.

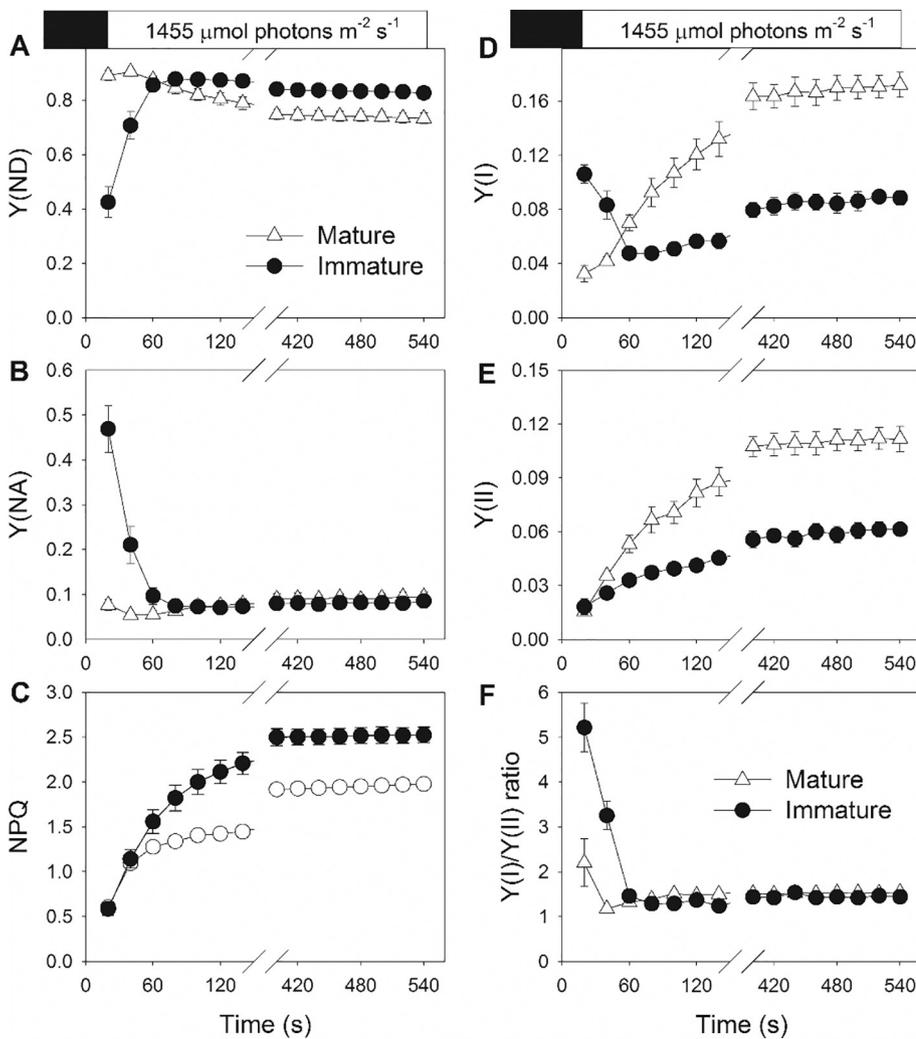
### 3.3. Changes in pmf and chloroplast ATP synthase activity after onset of light

In angiosperms, it is assumed that the *pmf* plays a critical role in the regulation of PSI redox state under fluctuating light. However, the changes in *pmf* components upon an increase in light intensity are little known. In order to understand the difference in PSI redox state upon dark-to-light transition between the mature and young leaves, the electrochromic shift (ECS) signal was measured to analyze the *pmf* components and chloroplast ATP synthase activity (Fig. S2). Upon transition from dark to a saturating light ( $1455 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), the *pmf* was rapidly generated for the first 20 s in both the mature and young leaves (Fig. 3A). Concomitantly, all leaves showed relative low levels of  $\Delta\text{pH}$  (Fig. 3B), owing to the large partitioning of *pmf* into  $\Delta\Psi$  (Fig. 3C). After this transition for 120 or 600 s, *pmf* and  $\Delta\Psi$  gradually decreased (Fig. 3A and C), but  $\Delta\text{pH}$  significantly increased (Fig. 3B). These results indicate that neither mature nor young leaves can generate a sufficient  $\Delta\text{pH}$  for the first seconds upon dark-to-light transition. The activity of chloroplast ATP synthase ( $g_{\text{H}^+}$ ) was strongly inhibited for the first 20 s after onset of the saturating light, and then gradually increased during illumination (Fig. 3D). After photosynthetic induction for 600 s, the mature leaves had significantly higher  $g_{\text{H}^+}$  than the young leaves.

### 3.4. Changes in PSI and PSII parameters under fluctuating light

We next examined the PSI and PSII performances under fluctuating light alternating between low/high light. PSI responses observed upon a sudden transition from low to high light were highly similar to the one detected in the dark-to-light transition presented earlier. During the transition from 59 to  $1455 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , PSI was highly oxidized for the first 10 s in the mature leaves (Fig. 4A), resulting in a low acceptor side limitation (Fig. 4B). By comparison, the young leaves showed much lower P700 oxidation ratio for the first 10 s (Fig. 4A), leading to the over-reduction of PSI electron carriers, as indicated by the high levels of Y(NA) (Fig. 4B). Therefore, the responses of PSI redox state to a sudden increase in light intensity largely differed between the mature and young leaves. After transition from low to high light, NPQ rapidly increased and reached the maximum values in 40 s (Fig. 4C), suggesting the fast formation of  $\Delta\text{pH}$ .

After transition from 59 to  $1455 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , Y(I) first decreased and then gradually increased in the mature leaves (Fig. 5A).



**Fig. 2.** Changes in PSI and PSII parameters during dark-to-light transition in mature and young leaves of *Bryophyllum pinnatum*. A, the quantum yield of non-photochemical energy dissipation due to the donor side limitation [Y(ND)]; B, the quantum yield of non-photochemical energy dissipation due to the acceptor side limitation [Y(NA)]; C, non-photochemical quenching in PSII; D, the quantum yields of PSI photochemistry [Y(I)]; E, the quantum yields of PSII photochemistry [Y(II)]; F, an indicator of CET activation [Y(I)/Y(II)]. After dark adaptation for at least 30 min, leaves were illuminated at 1455 μmol photons m<sup>-2</sup> s<sup>-1</sup> for 540 s and PSI parameters were measured every 20 s. Values are means ± SE (n = 5).

By comparison, in the young leaves, Y(I) gradually decreased to the minimum level (Fig. 5A). Y(II) showed a rapid decrease upon the increase in light intensity, and the mature leaves showed significantly higher Y(II) than the young leaves after this light transition for 2 min (Fig. 5B). Interestingly, for the first 10 s after transition from 59 to 1455 μmol photons m<sup>-2</sup> s<sup>-1</sup>, the Y(I)/Y(II) ratio increased from 0.9 to 1.0 to 1.5 and 2.6 in the mature and young leaves, respectively (Fig. 5C). After this transition for 120 s, the Y(I)/Y(II) ratio decreased to approximately 1.4 in both types of leaves (Fig. 5C). Furthermore, after transition from 1455 to 59 μmol photons m<sup>-2</sup> s<sup>-1</sup> for 2 min, the Y(I)/Y(II) ratio decreased to 0.9–1.0 in both the mature and young leaves. Because an increase in Y(I)/Y(II) is attributed to the activation of CET around PSI [2,3], these results suggested that the young leaves showed an increased cyclic electron transport upon transition from low to high light.

### 3.5. Changes in pmf and chloroplast ATP synthase activity under fluctuating light

The ECS signal was also measured under fluctuating light (Fig. 6). During the transition from 59 to 1455 μmol photons m<sup>-2</sup> s<sup>-1</sup>, the pmf performance was highly similar to the one detected in the dark-to-light transition (Fig. 3), with pmf in the first 10 s was significantly higher than that after transition for 120 s (Fig. 6A). Concomitantly, both the mature and young leaves showed insufficient ΔpH values for the first 10 s when compared with the ΔpH values after this light transition for 120 s (Fig. 6B). ΔΨ was generated to a relative high level in the first

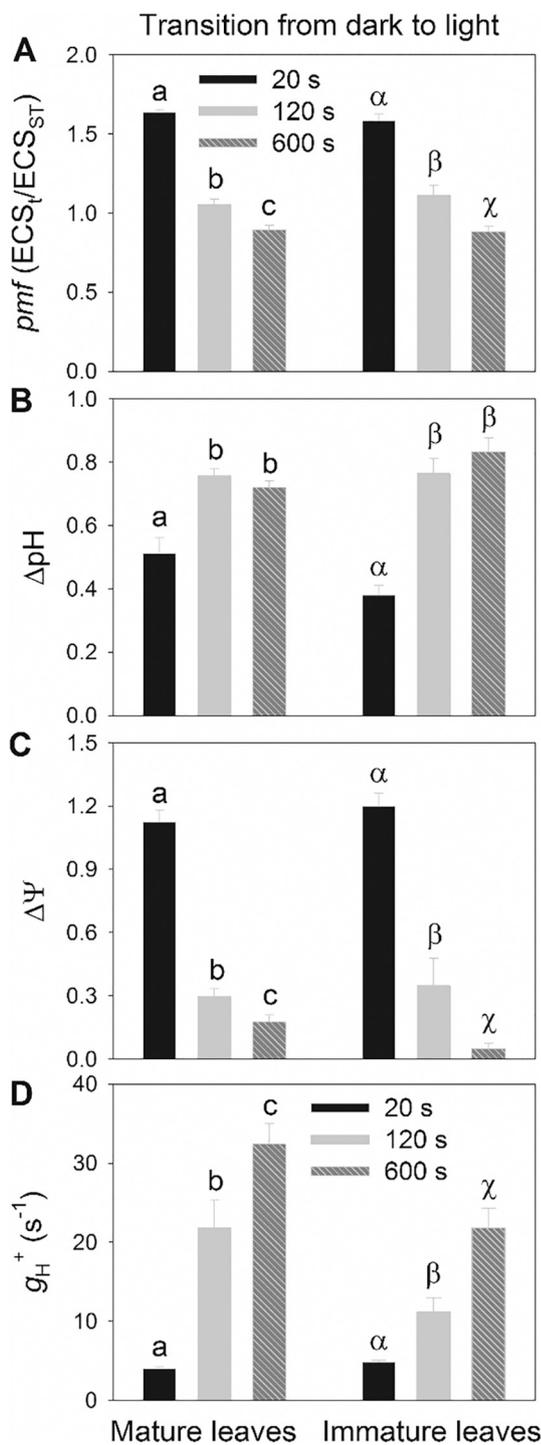
10 s and then significantly decreased (Fig. 6C). Interestingly, the change in g<sub>H</sub><sup>+</sup> during transition from low to high light differed between the mature and the young leaves (Fig. 6D). After this light transition, g<sub>H</sub><sup>+</sup> significantly increased in the mature leaves but was maintained stably in the young leaves.

### 3.6. Young leaves are more susceptible to PSI photoinhibition under fluctuating light

During the transition from 59 to 1455 μmol photons m<sup>-2</sup> s<sup>-1</sup> for 20 s, the over-reduction of PSI was observed in the young leaves but was absent in the mature leaves (Fig. 4). Because PSI can be damaged when PSI electron carriers are highly reduced under excess light energy, we speculate that 1) the fluctuating light can cause stronger PSI photoinhibition in the young leaves than the mature leaves; 2) the time point of PSI photoinhibition under fluctuating light is the first 20 s upon transition from low to high light. To test these hypotheses, mature and young leaves were exposed to fluctuating light alternating between 59 and 1455 μmol photons m<sup>-2</sup> s<sup>-1</sup> every 20 s. The results indicated that young leaves showed significantly stronger PSI photoinhibition during this fluctuating light treatment as compared with mature leaves (Fig. 7), further confirming the over-reduction of PSI within the first 20 s after transition from low to high light in young leaves.

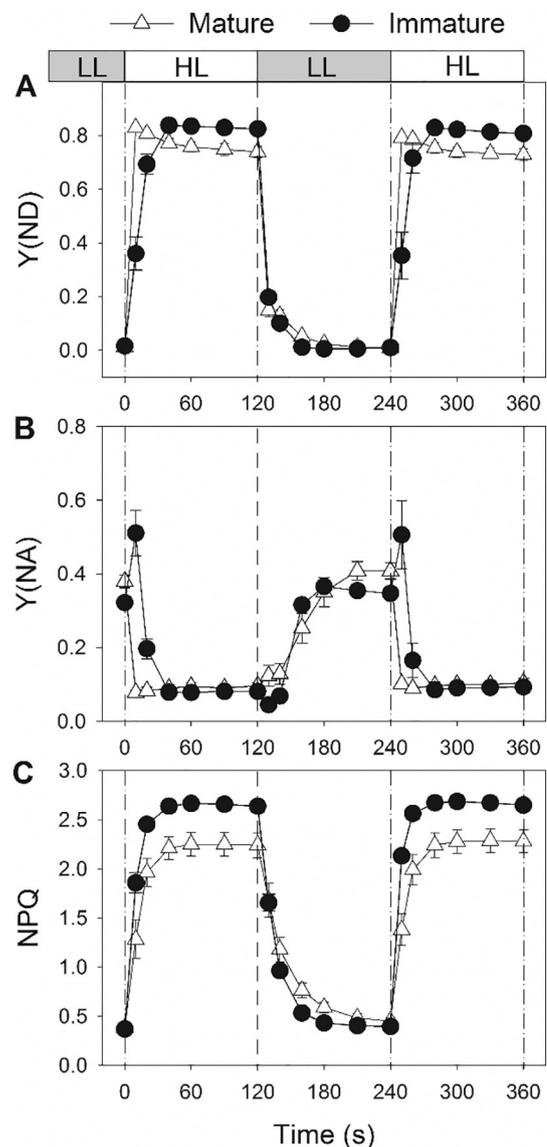
## 4. Discussion

Under natural field conditions, environmental conditions are highly



**Fig. 3.** ECS analysis during dark-to-light transition in mature and young leaves of *Bryophyllum pinnatum*. ECS signal was used to calculate the proton motive force ( $pmf$ ) (A), proton gradient ( $\Delta pH$ ) (B), membrane potential ( $\Delta \Psi$ ) (C) and proton conductivity ( $g_H^+$ ) across the thylakoid membranes (D). These parameters were measured after transition from dark to  $1455 \mu mol photons m^{-2} s^{-1}$  for 20 s, 120 s and 600 s. Values are means  $\pm$  SE ( $n = 5$ ). Different letters indicate significant differences among different treatments.

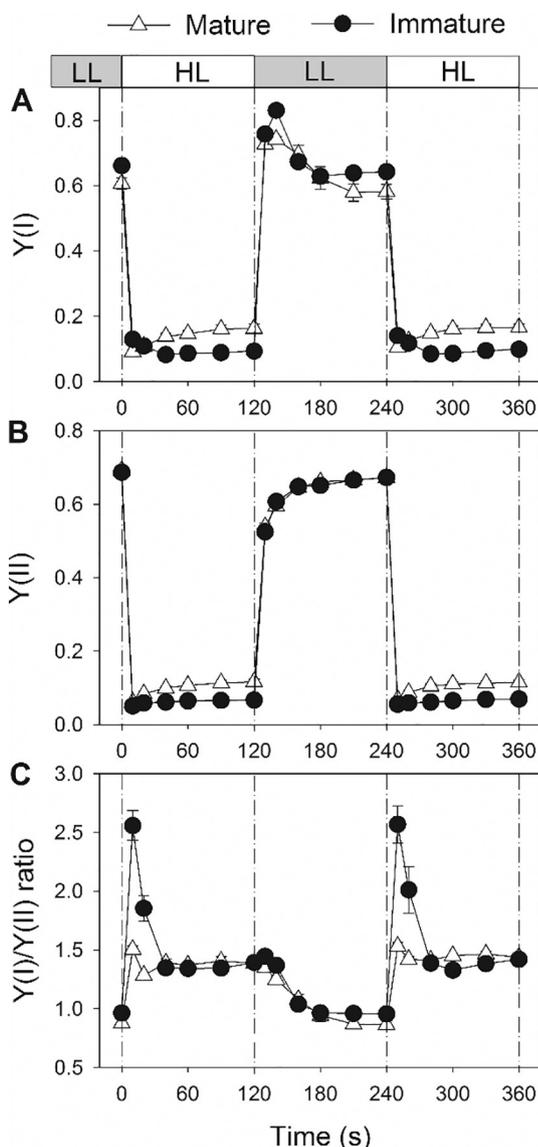
variable, and sudden changes in irradiation can induce the over-reduction of photosynthetic electron transport chains, leading to the generation of reactive oxygen species within thylakoid membranes, especially in PSI [11,14,39]. As a result, all oxygenic photosynthetic organisms must have strategies to protect PSI against photoinhibition under fluctuating light. The  $\Delta pH$ -dependent down-regulation of



**Fig. 4.** Changes in PSI redox state and NPQ under fluctuating light in mature and young leaves of *Bryophyllum pinnatum*. Before this analysis, leaves were illuminated at  $1455 \mu mol photons m^{-2} s^{-1}$  for 10 min, followed by exposure to  $59 \mu mol photons m^{-2} s^{-1}$  for 5 min. Afterward,  $Y(ND)$ ,  $Y(NA)$  and NPQ were measured under fluctuating light alternating between 59 and  $1455 \mu mol photons m^{-2} s^{-1}$ . These parameters were measured after light transition for 10 s, 20 s, 40 s, 60 s, 90 s and 120 s. Values are means  $\pm$  SE ( $n = 5$ ).

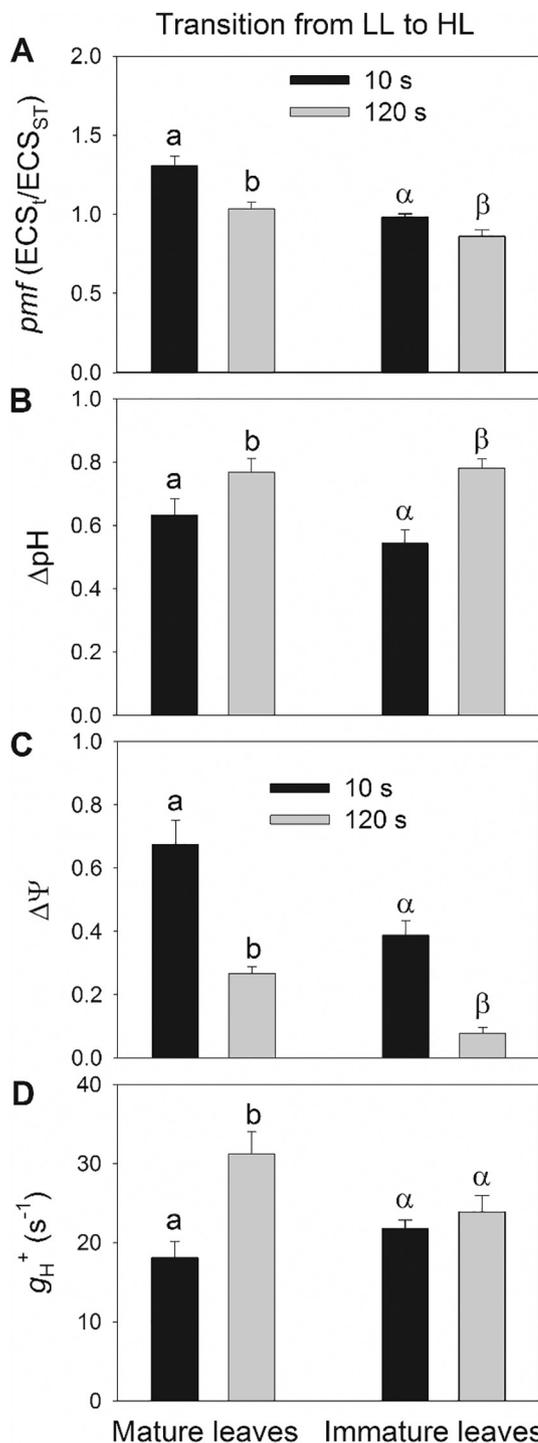
electron flow to PSI is thought to be the main mechanism enabling angiosperms growth under fluctuating light [7,11]. However, in angiosperms such as *Arabidopsis thaliana*, rice and *Bletilla striata* [41,43,44], PSI is over-reduced upon a sudden transition from low to high light, suggesting the deficiency of  $\Delta pH$ -dependent regulation of PSI redox state under fluctuating light in angiosperms. Some previous studies suggested that young leaves are more sensitive to PSI photo-inhibition under fluctuating light in *A. thaliana* [11], but the underlying mechanism is not well known. Furthermore, it is unclear how CAM plants deal with the rapid change in light intensity. Taken together, we here studied the photosynthetic regulation in mature and young leaves of the CAM angiosperm *B. pinnatum*, to attempt to address the above questions.

In the present study, we found that in the CAM angiosperm *B. pinnatum*, the P700 redox kinetics upon dark-to-light transition differed significantly between the mature and young leaves (Fig. 1). In the mature leaves, the re-oxidation of P700 was accomplished in 8 s. By



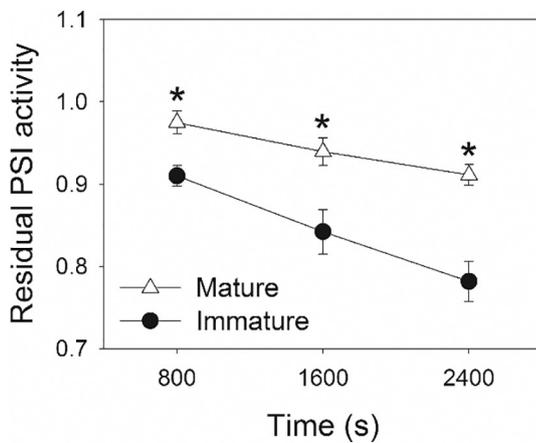
**Fig. 5.** Changes in quantum yields of photochemistry in PSI and PSII under fluctuating light in mature and young leaves of *Bryophyllum pinnatum*. Before this analysis, leaves were illuminated at  $1455 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 10 min, followed by exposure to  $59 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 5 min. Afterward, Y(I) and Y(II) were measured under fluctuating light alternating between 59 and  $1455 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . These parameters were measured after light transition for 10 s, 20 s, 40 s, 60 s, 90 s and 120 s. Values are means  $\pm$  SE (n = 5).

comparison, this fast re-oxidation of P700 was not observed in the young leaves (Fig. 1). In the P700 redox kinetics, the initial oxidation of P700 reflects the charge separation in P700 and electron transfer to ferredoxin. The subsequent P700 reduction reflects the electron transfer from PSII to PSI, and the final re-oxidation of P700 is attributed to the outflow of electrons from PSI to  $\text{O}_2$  [19]. In *A. thaliana* and young leaves of *B. pinnatum*, the fast re-oxidation of P700 was not observed (Fig. 1), indicating that CET-PSI cannot induce the fast re-oxidation of P700 upon dark-to-light transition. [20,21]. As a result, the fast PSI re-oxidation in the mature leaves of *B. pinnatum* should be caused by the operation of pseudoCET that is responsible for the outflow of electrons from PSI to  $\text{O}_2$ . PseudoCET includes two pathways: Flv-dependent pseudoCET and the water-water cycle (WWC) [36]. The former pathway contributes to the rapid re-oxidation of P700 in 1 s, which is not conserved in angiosperms [19]. Therefore, the fast re-oxidation of P700 in the mature leaves of *B. pinnatum* is attributed to the photo-



**Fig. 6.** ECS analysis under fluctuating light in mature and young leaves of *Bryophyllum pinnatum*. A, calculate the proton motive force (pmf); B, proton gradient ( $\Delta\text{pH}$ ); C, membrane potential ( $\Delta\Psi$ ); D, proton conductivity ( $g_{\text{H}^+}$ ) across the thylakoid membranes. These parameters were measured after transition from 54 to  $1455 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 10 s and 120 s. Values are means  $\pm$  SE (n = 5). Different letters indicate significant differences among different treatments.

reduction of  $\text{O}_2$  via the WWC. In the WWC, electrons are transported from PSI to  $\text{O}_2$ , leading to the photoreduction of  $\text{O}_2$  by the Mehler reaction [56]. Previous studies showed a significant contribution of the WWC to total electron flux in PSII in specific materials and experimental conditions [57–61]. Here, we showed that, in the mature leaves of *B. pinnatum*, the WWC was active as an electron sink downstream of



**Fig. 7.** Effect of fluctuating light on PSI activity in mature and young leaves of *Bryophyllum pinnatum*. After dark-adaptation for 30 min, leaves were exposed to fluctuating light alternating between 54 and 1455  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  every 20 s. Following treatments for 800, 1600 and 2400 min,  $P_m$  was determined after dark treatment for 3 min. Data were normalized to the initial values measured in the dark before the fluctuating light treatments. Values are means  $\pm$  SE ( $n = 5$ ). Asterisks indicate significant differences between the mature and young leaves.

PSI and of important for photosynthetic regulation during dark-to-light transition.

After transition from dark to a saturating light of 1455  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 20 s, PSI was highly oxidized in the mature leaves but was over-reduced in the young leaves (Fig. 2). Meanwhile, both the mature and young leaves could not generate a sufficient  $\Delta\text{pH}$  (Fig. 3B). As a result, the difference in PSI redox state between the mature and young leaves could not be explained by the  $\Delta\text{pH}$  formation. Furthermore, these results indicated that, for a few seconds after a sudden increase in illumination, the main player regulating PSI redox state differed between the mature and young leaves. In the young leaves, the  $\Delta\text{pH}$ -dependent down-regulation of electron flow at the Cyt  $b_6/f$  (photosynthetic control) is crucial for the oxidation of P700. By comparison, in the mature leaves, the WWC-dependent electron flow from PSI is prominent for the rapid oxidation of PSI electron carriers. After 1 min of light exposure, both the mature and young leaves formed enough  $\Delta\text{pH}$ , making the mature and young leaves indistinguishable. Therefore, during prolonged illumination with constant high light, the role of WWC in the regulation of PSI redox state is negligible, and the  $\Delta\text{pH}$ -dependent photosynthetic control protects PSI against photoinhibition in all leaves (Figs. 2 and 3).

The young leaves showed the over-reduction of PSI and relatively low  $\Delta\text{pH}$  upon a sudden transition from low to high light (Figs. 4 and 6). Furthermore, fluctuating light treatment caused significant PSI photoinhibition in the young leaves (Fig. 7). As a result, we propose that the PSI photoinhibition under fluctuating light is mainly caused by the insufficient  $\Delta\text{pH}$ . Upon a sudden increase in light intensity, electrons from PSII were accumulated in PSI. This resulting electron pressure induced production of superoxide and singlet oxygen within PSI [33], thus causing photoinhibition of PSI (Fig. 7). By comparison, in the mature leaves of *B. pinnatum*, the WWC activity as electron sink maintained PSI in a highly oxidation state after any increase in light intensity (Fig. 4). Because oxidized P700 ( $\text{P700}^+$ ) is a very good quencher of Chl excited states, PSI is extremely tolerant to excess excitation energy when P700 is high oxidized [11,22,62]. As a consequence, the WWC activity effectively adjusted PSI redox state and thus protected PSI from photodamage under fluctuating light in the mature leaves of *B. pinnatum*.

For the first seconds after an increase in illumination, young leaves showed an increased CET around PSI compared with the mature leaves. Thus, the reduced WWC activity in the young leaves is partially

compensated for by an increased CET, further suggesting that CET is the main player enabling young leaves growth under fluctuating light. In the *Arabidopsis* mutant lack the major pathway of CET, the *pgr5* mutant dies at the seedling stage when grown in fluctuating light conditions [39]. This lethality of *pgr5* in fluctuating light is thought to be caused by the lack of  $\Delta\text{pH}$ -dependent photosynthetic control at the Cyt  $b_6/f$  complex [11,63]. However, the relationship between  $\Delta\text{pH}$  formation and PSI redox state in young leaves is not well clarified. In this study, our results strongly indicated that in the young leaves of *B. pinnatum*, PSI redox state was indeed regulated mainly by the  $\Delta\text{pH}$ -dependent down-regulation of plastoquinol oxidation at the Cyt  $b_6/f$  complex.

In the young leaves of the CAM plant *B. pinnatum*, the insufficient  $\Delta\text{pH}$  resulted in the over-reduction of PSI upon a sudden increase in light intensity, which was also observed in the  $\text{C}_3$  plant *Arabidopsis thaliana* [45]. As a result, although photosynthetic regulation under fluctuating light in the young leaves of *B. pinnatum* is independent of stomatal conductance, the rapid increase in light intensity also generates a dangerous imbalance between electron flow from PSII and consumption of the final products of photosynthetic electron flow. Upon a sudden increase in illumination, the thylakoid lumen acidification is relatively weak, resulting in the rapid increase in electron transport from PSII to PSI. Meanwhile, the regulation of carbon fixation and other primary metabolism have slower kinetics, and thus they cannot immediately consume all the ATP and reducing power produced by photosynthetic electron flow. As a result, the electron transport from PSI to  $\text{NADP}^+$  would rapidly become limiting by the lack of  $\text{NADP}^+$ , causing the accumulation of reduced electron carriers in PSI and initiating PSI photoinhibition.

## 5. Conclusions

In summary, here we have highlighted the photosynthetic regulation under fluctuating light between the mature and young leaves of the CAM plant *B. pinnatum*. We found that in the mature leaves, the WWC optimized PSI redox state after any increase in light intensity, thus preventing PSI photoinhibition under fluctuating light. By comparison, the PSI redox state in the young leaves was mainly controlled by the  $\Delta\text{pH}$ . Upon a sudden increase in illumination, the insufficient  $\Delta\text{pH}$  made PSI highly reduced in the young leaves, leading to PSI photoinhibition under fluctuating light. These results strongly indicated that photosynthetic regulation under fluctuating light was affected by developmental stages. Furthermore, we demonstrate that, in addition to  $\Delta\text{pH}$ -dependent photosynthetic control, the WWC activity plays an important role in photoprotection for PSI under fluctuating light.

## Conflict of interest

The authors declare no conflict of interest.

## Transparency document

The Transparency document associated with this article can be found, in online version.

## Acknowledgements

This work is supported by the National Natural Science Foundation of China (Grant 31670343), the Youth Innovation Promotion Association of the Chinese Academy of Sciences (Grant 2016347).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbabi.2019.04.006>.

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