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Role of cyclic electron transport around photosystem I in regulating proton motive force☆

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ABSTRACT

In addition to ΔpH formed across the thylakoid membrane, membrane potential contributes to proton motive force (*pmf*) in chloroplasts. However, the regulation of photosynthetic electron transport is mediated solely by ΔpH. To assess the contribution of two cyclic electron transport pathways around photosystem I (one depending on PGR5/PGRL1 and one on NDH) to *pmf* formation, electrochromic shift (ECS) was analyzed in the *Arabidopsis pgr5* mutant, NDH-defective mutants (*ndhs* and *crr4-2*), and their double mutants (*ndhs pgr5* and *crr4-2 pgr5*). In *pgr5*, the size of the *pmf*, as represented by ECS_t, was reduced by 30% to 47% compared with that in the wild type (WT). A g_H^+ parameter, which is considered to represent the activity of ATP synthase, was enhanced at high light intensities. However, g_H^+ recovered to its low-light levels after 20 min in the dark, implying that the elevation in g_H^+ is due to the disturbed regulation of ATP synthase rather than to photodamage. After long dark adaptation more than 2 h, g_H^+ was higher in *pgr5* than in the WT. During induction of photosynthesis, g_H^+ was more rapidly elevated in *pgr5* than that in the WT. Both results suggest that ATP synthase is not fully inactivated in the dark in *pgr5*. In the NDH-deficient mutants, ECS_t was slightly but significantly lower than in the WT, whereas g_H^+ was not affected. In the double mutants, ECS_t was even lower than in *pgr5*. These results suggest that both PGR5/PGRL1- and NDH-dependent pathways contribute to *pmf* formation, although to different extents. This article is part of a Special Issue entitled: Chloroplast Biogenesis.

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1. Introduction

In the thylakoid membranes of chloroplasts, light-driven electron transport is coupled with the establishment of a proton (H^+) gradient (ΔpH). ΔpH, together with the membrane potential (Δψ), constitutes the total proton motive force (*pmf*), which is ultimately used to drive chloroplast F_0F_1-ATP synthase (ATP synthase). Whereas both components of the *pmf* contribute to ATP synthesis [1,2], only ΔpH can induce the energization-dependent quenching (qE) component of non-photochemical quenching (NPQ) of chlorophyll fluorescence via acidification of the thylakoid lumen [3,4]. Through this qE mechanism, absorbed excessive light energy is safely dissipated as heat from the light-harvesting complexes of photosystem (PS) II. In Mitchell's hypothesis, ΔpH and Δψ are thermodynamically and kinetically equivalent [5,6]. In the mitochondria, almost all of the *pmf* is stored in the form of Δψ because of the low permeability of the mitochondrial inner membrane to ions. In contrast, studies in electrode-impaled giant chloroplasts have indicated that virtually all of the Δψ component of the *pmf* is rapidly dissipated under continuous illumination [7–9], suggesting that the contribution of ΔpH to *pmf* predominates in chloroplasts. Kramer and co-workers argued against this idea by demonstrating that a substantial fraction of *pmf* can be stored as Δψ during steady-state photosynthesis [10,11]. However, a recent study using specific inhibitors of ΔpH and Δψ formation and a mutant lacking the induction of qE came to a different conclusion [12].

The electrochromic shift (ECS) is a bandshift phenomenon in the absorption spectra of some photosynthetic pigments and depends on the presence of an electric field formed across the thylakoid membrane [1]. ECS can be used to probe the trans-thylakoid *pmf* in intact leaves [13]. During steady-state photosynthesis, the size of the *pmf* is determined by the balance between its generation, which depends on photosynthetic electron transport, and its relaxation, which depends mainly on ATP synthase activity [14]. Applying a dark pulse by switching off the actinic light for 1 s during steady-state photosynthesis collapses the light-dependent H^+ influx into the thylakoid

Abbreviations: AL, actinic light; ECS, electrochromic shift; ETR, electron transport rate; Fd, ferredoxin; NDH, NADH dehydrogenase-like complex; NPQ, non-photochemical quenching; PGR5, PROTON GRADIENT REGULATION 5; PGRL1, PGR5-LIKE PHOTOSYNTHETIC PHENOTYPE 1; *pmf*, proton motive force; PS, photosystem; Φ_{PSII}, PSII quantum yield; $P700^+$, oxidized PSI reaction center; PQ, plastoquinone; qE, energization-dependent NPQ

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lumen and can thus be used to elucidate the rate of H^+ efflux via ATP synthase [15]. The amplitude of the light-dark difference in the ECS signal (ECS_t) represents the total size of the *pmf* formed in the light. In addition, the H^+ conductivity of ATP synthase (g_H^+) can be calculated from the kinetics of the initial ECS decay.

Formation of *pmf* depends on both linear and PSI cyclic electron transport. In linear electron transport, *pmf* formation is coupled with O_2 evolution and reduction of $NADP^+$. In contrast, PSI cyclic electron transport generates *pmf* without net accumulation of NADPH by recycling electrons from ferredoxin (Fd) or NADPH to the plastoquinone (PQ) pool [16]. In *Arabidopsis*, PSI cyclic electron transport consists of two partly redundant pathways [17]: The main pathway depends on PGR5 (PROTON GRADIENT REGULATION 5) and PGRL1 (PGR5-LIKE PHOTOSYNTHETIC PHENOTYPE) proteins [18,19] and is sensitive to antimycin A [20,21]. The PGR5/PGRL1-dependent pathway likely corresponds to PSI cyclic electron transport that was discovered by Arnon and co-workers [22]. The other pathway is insensitive to antimycin A and is mediated by the chloroplast NADH dehydrogenase-like (NDH) complex [23–25]. In the *pgr5* mutant, induction of qE is severely impaired, suggesting that PGR5/PGRL1-dependent PSI cyclic electron transport contributes markedly to ΔpH formation, which is needed to induce qE [18,26]. In contrast, a defect in chloroplast NDH does not affect qE induction [23,24]. Despite the minor contribution of chloroplast NDH to qE, double mutants defective in the two PSI cyclic electron transport pathways have severe phenotypes of photosynthesis and plant growth [17]. These results suggest the important role of NDH-dependent PSI cyclic electron transport in the *pgr5* mutant background. How does chloroplast NDH alleviate the *pgr5* phenotype? The exact molecular mechanism is still unclear.

The *pgr5* mutant is defective in *pmf* formation via PSI cyclic electron transport [17,18,26]. ECS analysis has estimated that 13% of ATP output in *Arabidopsis* depends on PGR5/PGRL1-dependent PSI cyclic electron transport [27]. Unexpectedly, however, ECS analysis has also revealed that g_H^+ is enhanced in the *pgr5* mutant [27]. Upregulation of ATP synthase may compensate partially for the reduced ATP synthesis, but it should seriously disturb the regulation of photosynthetic electron transport that is induced via low lumen pH at high light intensities in the wild type (WT) [17,18,26,28,29]. Neither the molecular mechanism nor the physiological meaning of the g_H^+ phenotype of the *pgr5* mutant is clear. In this study, we extended the ECS analysis of *Arabidopsis* mutants defective in PSI cyclic electron transport to further characterize the mysterious g_H^+ phenotype of the *pgr5* mutant and to directly analyze the contribution of chloroplast NDH to *pmf* in the *pgr5* mutant background.

2. Material and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana WT (ecotype Columbia *gl1*) and mutants were grown in soil for 8 to 12 weeks under growth chamber conditions (50 μmol photons m^{-2} s^{-1}, 8 h light/16 h dark cycles at 23 °C).

2.2. Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters were measured with a MINI-PAM (pulse-amplitude modulation) portable chlorophyll fluorometer (Walz, Effeltrich, Germany) in ambient air at room temperature (25 °C), as described previously [30]. NPQ and the quantum yield of PSII (Φ_{PSII}) were calculated as $(Fm - Fm')$ / Fm' and $(Fm' - Fs)$ / Fm', respectively. Relative ETR was calculated as $\Phi_{PSII} \times$ PFD (photon flux density).

2.3. ECS 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). Plants were first dark adapted for 15 min and then the detached leaves were analyzed. Except in the analysis in Fig. 3, the ECS signal was obtained after 2 to 3 min of illumination at different actinic light (AL) intensities, and a 1-s dark pulse was applied three times (30 s apart) for technical replicates. In Fig. 2C and Supplementary Fig. S2, longer illumination of AL (15 to 16 min) was used to record steady-state ECS signals. ECS_t, which represents the difference in total *pmf* between light and dark, 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 515-nm absorbance change induced by a single turnover flash (ECS_{ST}), as measured on dark-adapted leaves before recording. This normalization accounted for changes in leaf thickness and chloroplast density between leaves [31]. g_H^+, which reflects the proton conductivity of ATP synthase, was estimated by fitting the first 300 ms of the decay curve with a first-order exponential decay kinetic as the inverse of the decay time constant, as described by Avenson et al. [27].

3. Results

3.1. Effects of PGR5-dependent PSI cyclic electron transport on formation and relaxation of pmf

ECS_t (ECS_t /ECS_{ST}) represents the total size of the *pmf* formed in the light. The contribution of PGR5-dependent PSI cyclic electron transport to *pmf* formation is supported directly by a lower ECS_t observed in the *Arabidopsis pgr5* mutant [27] and in rice *PGR5* knockdown lines [26]. To analyze the impact of the *pgr5* defect on *pmf* in a broader range of light intensities (46 to 1076 μmol photons m^{-2} s^{-1}), the light-intensity dependence of ECS signal parameters were compared between WT and *pgr5* mutant plants (Fig. 1). Consistent with the findings of a previous report by Avenson et al. [27], ECS_t was dramatically reduced in *pgr5* plants at light intensities of 138 μmol photons m^{-2} s^{-1} or more (Fig. 1A). As in rice [26], however, the difference was subtle at a low light intensity of 46 μmol photons m^{-2} s^{-1}, consistent with the normal plant growth at 50 μmol photons m^{-2} s^{-1} [32]. In addition, an increase in the ECS decay rate (g_H^+), which likely reflects the H^+ conductivity of chloroplast ATP synthase, is observed in the *Arabidopsis pgr5* mutant [27]. Consistently, we observed more rapid decay of ECS signals in *pgr5* than in the WT at high light intensities (Supplementary Fig. S1), from which g_H^+ was calculated. At light intensities of 320 μmol photons m^{-2} s^{-1} or more we observed a significant increase in g_H^+, reflecting increased H^+ conductivity of ATP synthase [27], in *pgr5* mutants compared with the WT, whereas g_H^+ changed little in the WT (Fig. 1B). Faster proton efflux from the lumen through the ATP synthase, as indicated by higher g_H^+, partly explains the lower *pmf* in *pgr5* at light intensities higher than 320 μmol photons m^{-2} s^{-1}. At 138 μmol photons m^{-2} s^{-1}, however, the small elevation in g_H^+ cannot solely explain the drastic reduction in ECS_t observed in *pgr5* mutants (Fig. 1).

In Fig. 1, the leaves were exposed to actinic light (AL) at each light intensity for 2 to 3 min. Because three dark pulses were applied at each AL intensity, the first dark pulse was after 2-min and the last one was after 3-min exposure of AL. Induction of the Calvin cycle may influence the ECS signal in this period after long dark adaptation. To assess this possibility, ECS signals were compared after short (2 to 3 min) and long (15 to 16 min) AL exposure (Supplementary Fig. S2). At 138 μmol photons m^{-2} s^{-1}, ECS_t was slightly lower after a long AL exposure than after a short AL exposure in both the WT and *pgr5*, and this process was accompanied by a slight increase in g_H^+. The 2 to 3-min AL exposure may not be long enough to monitor the steady-state levels of g_H^+ at 138 μmol photons m^{-2} s^{-1}, which was not statistically different between *pgr5* and the WT (Supplementary Fig. S2D). This is inconsistent with an independent measurement, in which g_H^+ was slightly but significantly higher in *pgr5* than that in the WT (Fig. 1B). The difference between the genotypes was not statistically significant after long

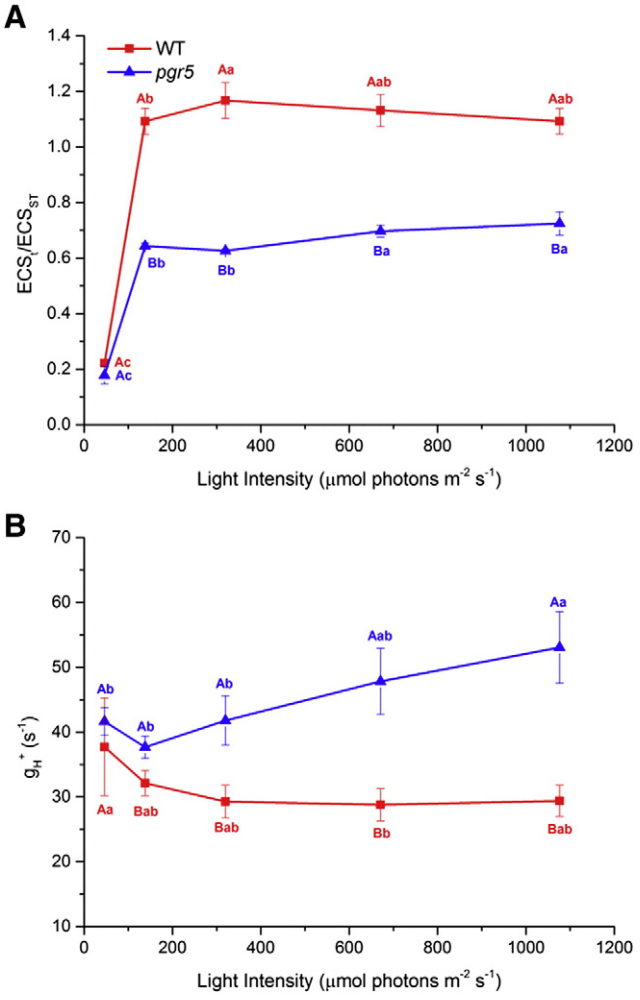

Fig. 1. ECS analysis of the wild type (WT) and the *pgr5* mutant. For each AL intensity, a 1-s dark pulse was applied three times (technical replicates) and the averages were calculated. Data represent means ± SD (n = 3–4 of biological replicates with 3 technical replicates). The different uppercases represent statistically significant difference between genotypes at the same light intensity, whereas the lowercases represent that among light intensities in the same genotype ($p < 0.05$, one-way ANOVA). (A) Light-intensity dependence of ECS_t. The ECSt levels were standardized against the 515-nm absorbance change induced by a single turnover flash (ECS_{ST}). (B) Light-intensity dependence of g_H^+, which was estimated from a decay kinetics of the ECS signal on an abrupt light-dark transition.

AL exposure (Supplementary Fig. S2D). The light intensity of 138 μmol photons m^{-2} s^{-1} may be marginal to induce elevated g_H^+ in *pgr5*. Consistent with the original idea that PSI cyclic electron transport is affected in *pgr5* mutants [18], these results suggest that *pmf* formation was partly suppressed in *pgr5* mutants. Additionally, this defect was accompanied by enhanced H^+ efflux from the lumen, especially at high light intensities.

The *pgr5* defect causes a reduction in NPQ as a consequence of reduced ΔpH [18,26]. We cultured *Arabidopsis* seedlings under short-day conditions because the ECS analysis technically required larger leaves. We analyzed the light-intensity dependence of the Chl fluorescence parameters NPQ and relative electron transport rate (ETR) in PSII in the plants used for the ECS analysis (Supplementary Fig. S3). As observed in plants cultured under long-day conditions [18], NPQ induction was impaired at high light intensities in the *pgr5* mutants (Supplementary Fig. S3A). The relative ETR of PSII was saturated at a lower level in the *pgr5* mutants than in the WT, although ETR was not affected at low light intensities (less than 100 μmol photons m^{-2} s^{-1}; Supplementary Fig. S3B). *pmf* was saturated at 138 μmol photons m^{-2} s^{-1} in both the WT (ECS_t/ECS_{ST} = ~1.1) and the *pgr5* mutant (ECS_t/ECS_{ST} = 0.6–0.7) (Fig. 1A), but NPQ still increased in the course of an increase in light intensity to 138 μmol photons m^{-2} s^{-1} or more (Supplementary Fig. S3A).

3.2. Regulation of chloroplast ATP synthase is more sensitive to light in pgr5 mutants than in the WT

The ECS parameter g_H^+ is considered to represent the H^+ conductivity of the thylakoid membrane, probably via ATP synthase. Puzzlingly, g_H^+ was enhanced in the *pgr5* mutant, although *pmf* formation was reduced [26,27]. This finding may reflect the activation of ATP synthase to compensate for the reduced ATP level [26,29]. Because the steady-state level of *pmf* is determined by the balance between its generation and relaxation, in *pgr5* mutants the activation of ATP synthase may be accompanied by disturbed regulation of photosynthetic electron transport via increased pH of the thylakoid lumen at high light intensities. Indeed, *pgr5* plants exhibited defects in NPQ induction [18] and in downregulation of the cytochrome b_6f complex when exposed to high light [28]. An increase in g_H^+ may result from damage caused to the thylakoid membrane by high light intensities rather than from disturbance of the regulation of ATP synthase, as the *pgr5* mutant is sensitive to high light intensity [18,30]. To test these possibilities, we monitored the recovery of high light-induced high g_H^+ levels in the dark (Fig. 2). Plants were kept dark-adapted for 15 min and then detached leaves were exposed to low light of 138 μmol photons m^{-2} s^{-1}, at which level the ECS_t was significantly lower in the *pgr5* mutant than in the WT (Fig. 2A, before). As observed in Supplementary Fig. S2D, the difference in g_H^+ was not significant between the WT and the *pgr5* mutant at the same low light intensity (Fig. 2B, before). The leaves were then exposed to high-intensity light of 1076 μmol photons m^{-2} s^{-1} to induce an increase in g_H^+ in the *pgr5* mutant (Fig. 2B, 0 min). Subsequently, the leaves were kept in the dark to monitor changes in ECS signals for 6 h to test whether g_H^+ recovered to the low-light levels (Fig. 2). As a control, detached WT leaves were not exposed to high light and kept in the dark during the whole dark adaptation. To monitor ECS, the dark period was interrupted by a short period of exposure to low light (138 μmol photons m^{-2} s^{-1} for 2 to 3 min in Fig. 2A, B). After dark adaptation of the leaves for 20 min, g_H^+ in the *pgr5* mutant recovered to its pre-high-light treatment level (Fig. 2B). As observed after a long period of dark adaptation (Fig. 2B, more than 120 min), the g_H^+ at 138 μmol photons m^{-2} s^{-1} depended on the length of the dark adaptation before measurement. Notably, the WT showed the same fluctuation of g_H^+ levels as *pgr5*, although the amplitude of its changes was smaller (Fig. 2B). Control WT leaves in the dark behaved like the illuminated WT leaves, except that they did not show the transient response to high light (Fig. 2A, B).

To monitor the fluctuation of ECS signals in the short period, we used short exposure time (2 to 3 min) of AL in Fig. 2A and B, as in Fig. 1. To assess the possibility that longer exposure of AL for determining ECS parameters affects the conclusion, 15 to 16-min AL was also used in some time points (Fig. 2C). In general, longer exposure of AL slightly increased g_H^+ in both the WT and *pgr5* at all time points, resulting in the parallel fluctuation of g_H^+ between two different AL exposure periods. A substantial difference depending on the different exposure time of AL was observed only just after the treatment to high light (time 0), at which g_H^+ was higher in *pgr5* mutants than that in the WT in the 2 to 3-min measurement but was not statistically different in the 15 to 16-min measurement (Fig. 2C). It is possible that recovery of g_H^+ already started at 138 μmol photons m^{-2} s^{-1} for 15 to 16 min after high light treatment in *pgr5* mutants.

Because the high g_H^+ level induced by high light recovered to its low-light level over a relatively short period, this g_H^+ phenotype in the *pgr5* mutant likely reflects a regulatory process rather than photodamage to the thylakoid membrane. After prolonged dark adaptation for 120 min or more, g_H^+ levels in the WT were even lower than the original levels, whereas the levels in the *pgr5* mutant were still higher than in

the WT (Fig. 2B). This suggests that, unlike in the WT chloroplast, ATP synthase in *pgr5* mutants is not fully deactivated in the dark. We also monitored the changes in ECS_t, which fluctuated in a way opposite to g_H^+ (Fig. 2A; Supplementary Fig. S4). As observed in Fig. 1, the difference in ECS_t before high light treatment cannot be fully explained by the difference in g_H^+, but the changes of ECS_t were opposite to those of g_H^+ after high light treatment in both the WT and the *pgr5* mutant (Fig. 2; Supplementary Fig. S4). On the basis of these results, we conclude that the enhanced g_H^+ observed at high light intensities in the *pgr5* mutant reflects the disturbed regulation of *pmf*, which probably resulted from the regulation via chloroplast ATP synthase rather than photodamage. Notably, a similar pattern of response but with lower amplitude was observed in the WT, suggesting that the *pgr5* defect elevated the sensitivity of the regulation of ATP synthase, which operated also in the WT. It may be necessary to consider different physiological reasons for the higher g_H^+ observed after long dark adaptation (120 min or more) and for the higher g_H^+ observed after relatively short dark adaptation (<20 min) in the *pgr5* mutant (see Discussion).

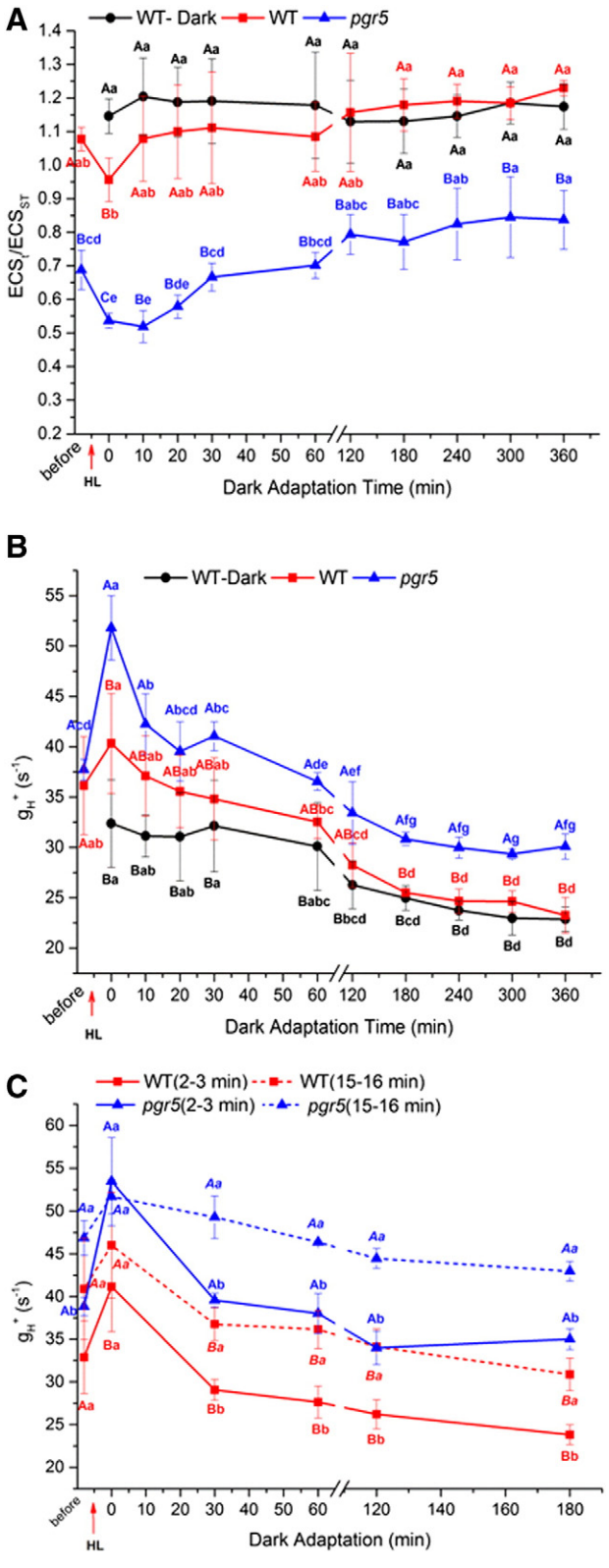

Fig. 2. Recovery of total ECS (A) and g_H^+ (B and C) over 6 h (A and B) or 3 h (C) in the dark after a 3.5-min high light exposure (red arrows). Leaves were pre-illuminated at 138 μmol photons m^{-2} s^{-1} and a 1-s dark pulse was applied to monitor ESC signals before the high light treatment (Fig. 2, before). Subsequently, the leaves were exposed to high light of 1078 μmol photons m^{-2} s^{-1} for 3.5 min, followed by long dark adaptation (6 h or 3 h) to monitor the recovery of the ECS signals. "0" represent the ECS signals measured immediately after the high light treatment. During the recovery phase in the dark, low-intensity AL of 138 μmol photons m^{-2} s^{-1} was applied again to monitor the ECS signals at each time point. At the end of each low-light period, a 1-s dark pulse was applied three times (technical replicates) and the averages were calculated. ECS_t and g_H^+ were calculated as described in Fig. 1. Data represent means ± SD (n = 3 biological replicates with 3 technical replicates). In A and B, the different uppercases represent statistically significant difference between genotypes at the same time point, whereas the lowercases represent that among time points in the same genotype or treatment ($p < 0.05$, one-way ANOVA). In C, at the same time point, the different uppercases represent statistically significant difference between genotypes at the same AL period, whereas the lowercases represent that between different AL periods in the same genotype ($p < 0.05$, one-way ANOVA).

3.3. pgr5 defect accelerates light-induced activation of chloroplast ATP synthase

In the *pgr5* mutant, the sensitivity of chloroplast ATP synthase to high light was exaggerated, and the activity of the enzyme was not fully downregulated in the dark (Fig. 2). To further study the link between the PGR5/PGRL1-dependent PSI cyclic electron transport and regulation of ATP synthase, we attempted to assay the light-induced activation of ATP synthase by monitoring ECS during the induction of photosynthesis (Fig. 3). Before the analysis, leaves were dark adapted for 15 min and then exposed to low light of 138 μmol photons m^{-2} s^{-1}. During the induction of photosynthesis for 2 min, 1-s dark pulses were applied to monitor ECSt and g_H^+. After the initial 10 s, g_H^+ was similar in the WT and in the *pgr5* mutant, whereas ECS_t was significantly lower in the mutant, suggesting that PGR5-dependent PSI cyclic electron transport was operating in the WT (Fig. 3). In the WT, g_H^+ was only slightly upregulated in the first 60 s of illumination, and it took 90 s to activate ATP synthase to the steady-state level (Fig. 3B). In contrast, in the *pgr5* mutant, g_H^+ was significantly enhanced after 20 s of illumination and was saturated after 60 s at a higher level than in the WT, suggesting that ATP synthase was activated more rapidly in the mutant. Opposite to the increase in g_H^+, ECS_t decreased from 1.14 to 0.68 in the *pgr5* mutant after 2 min of illumination, whereas this process happened more slowly and to a lesser extent (from 1.50 to 1.17) in the WT (Fig. 3A). We observed similar ECS phenotypes when we used a lower light intensity (46 μmol photons m^{-2} s^{-1}) (data not shown). Although we cannot exclude the possibility of collapse of a fraction of the $\Delta\psi$ component because of counterion movement during the induction of photosynthesis [11], the observed decline in ECS_t can be explained largely by an increase in g_H^+. ATP synthase is activated more rapidly and to a greater extent in the *pgr5* mutant during the induction of photosynthesis.

3.4. ECS analysis in two NDH mutants and their double mutants

The PGR5/PGRL1-dependent PSI cyclic electron pathway contributed markedly to the regulation of *pmf* (Figs. 1 and 2). Consistently, the *pgr5* defect has pleiotropic effects on photosynthesis in leaves [32]. The defect in chloroplast NDH does not influence photosynthesis in

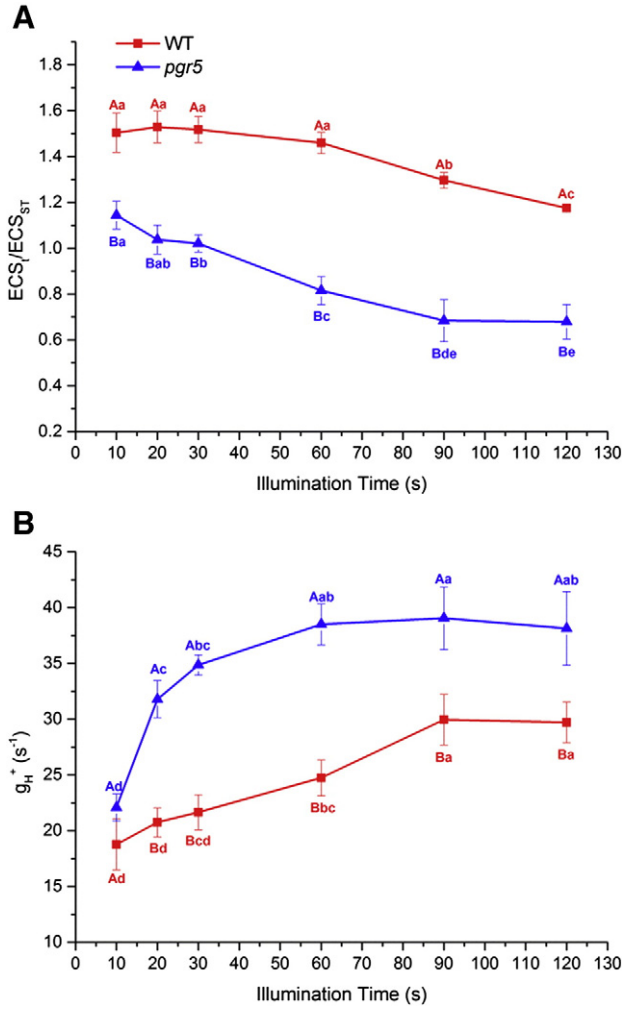

Fig. 3. Fluctuations in ECS_t (A) and g_H^+ (B) during the induction of photosynthesis at 138 μmol photons m^{-2} s^{-1}. A single dark pulse was applied at each period of induction. Data represent means ± SD ($n = 3$ biological replicates). Different uppercases represent statistically significant difference between genotypes at the same time point, whereas lowercases represent that among time points in the same genotype ($p < 0.05$, one-way ANOVA).

flowering plants [13,17,24]. However, a severe phenotype is observed in double mutants lacking both pathways of PSI cyclic electron transport [17]. This clear genetic evidence for the interaction of two PSI cyclic pathways has not been fully explained by physiological study. The rate of NDH-dependent PSI cyclic electron transport was estimated to be lower than that of PGR5/PGRL1-dependent electron transport [17]. How does the NDH-dependent electron transport rescue the *pgr5* defect? To address this question, we used *ndhs*/*crr31* and *crr4-2* mutants defective in chloroplast NDH, along with their double mutants with *pgr5* (*ndhs pgr5-1* and *crr4-2 pgr5-1*) (Fig. 4). NdhS is an NDH subunit required for the high-affinity binding of ferredoxin to NDH [33,34], whereas the *crr4-2* mutant is defective in a pentatricopeptide repeat (PPR) protein required for the RNA editing that creates the translational initiation codon of *ndhD* [35]. For technical reasons, we selected these mutants with relatively good growth in the *pgr5* mutant background, as they have either leaky activity (*ndhS*) or accumulation (*crr4-2*) of NDH [17,33]. Despite the drastic decrease in *pmf* size in the *pgr5* mutant, the decrease in ECS_t was slight but significant in the *ndhS* and *crr4-2* mutants (Fig. 4A, C). The level of g_H^+ was not affected at any light intensity in either NDH mutant (Fig. 4B, D). Consistent with the severe photosynthetic phenotype in the *pgr5* mutant background [17,33], ECS_t was significantly lower in the double mutants than in the single NDH mutants (Fig. 4A, C). Because the rate of linear electron transport is also reduced in the double mutants [17,33], all of the decrease in *pmf* in the double mutants cannot be ascribed to NDH deficiency. However, taken together with the results of our analysis of the single mutants, these results provide direct evidence for the contribution of NDH to *pmf* formation. Neither the *ndhS* nor the *crr4-2* defect affected the level of g_H^+, suggesting that the NDH defect enhanced the ECS_t phenotype of *pgr5* mutants but was not associated with changes to the regulation of H^+ conductivity of ATP synthase (Fig. 4B, D).

4. Discussion

4.1. Distinct contribution of two PSI cyclic electron transport pathways to pmf formation

We directly investigated the contribution of PSI cyclic electron transport to *pmf* by analyzing ECS_t in the *pgr5* mutant, two NDH mutants (*ndhs* and *crr4-2*), and their double mutants with *pgr5*. At a low light intensity of 46 μmol photons m^{-2} s^{-1}, ECS_t was similar in the *pgr5* mutant and the WT, whereas up to 40% reduction was observed in the mutant when light intensity was elevated above 138 μmol photons m^{-2} s^{-1} (Fig. 1A). Consistently, the *pgr5* mutant grows like the WT at 50 μmol photons m^{-2} s^{-1}, but a slight increase in light intensity to 150 μmol photons m^{-2} s^{-1} drastically affects biomass production (60% reduction) in the mutant [32]. The suppression in relative ETR in the *pgr5* mutant at 320 μmol photons m^{-2} s^{-1} was more evident than that at 138 μmol photons m^{-2} s^{-1} (Supplementary Fig. S3B), whereas the reductions in ECS_t in the mutant were comparable at these two light intensities (40% and 46% at 138 and 320 μmol photons m^{-2} s^{-1}, respectively) (Fig. 1A). The disturbed ratio of ATP/NADPH production in *pgr5* may be compensated for at low light intensities (less than 138 μmol photons m^{-2} s^{-1}) by other alternative electron transport pathways. However, they likely did not work sufficiently at higher light intensities to supply more ATP and consequently rescue the shortage of $NADP^+$, resulting in the saturation of ETR at lower levels in *pgr5* (Supplementary Fig. S3B). These results suggest that PGRL1/PGR5-dependent cyclic electron transport contributes to *pmf* formation at a wide range of light intensities but becomes more important at higher light intensities.

In contrast to the case in the *pgr5* mutant, *crr* mutants defective in chloroplast NDH did not show any mutant phenotype under growth chamber conditions. However, the *crr* defect drastically enhances the *pgr5* mutant phenotype [17], although the exact mechanism for this synergistic effect is unclear. In this study, we showed that, in the double mutants, ECS_t was reduced more than in the *pgr5* single mutant (Fig. 4A, C), suggesting that chloroplast NDH contributes markedly to *pmf* formation in the *pgr5* mutant background. Although the rate of linear electron transport is also reduced in the double mutants [17,33], we observed that ECS_t was slightly but significantly reduced in both the *ndhs* and the *crr4-2* mutant (8% to 12% and 8% to 14%, respectively) (Fig. 4A, C). This is consistent with the fact that $P700^+$ levels are slightly reduced in *crr* single mutants [17]. Even the slight decrease in *pmf* likely affects downregulation of the cytochrome b_6f complex and electron acceptance from PSI, resulting in a reduction in $P700^+$ levels in the *crr* single mutants. Although the extents of their contributions differ, both PGR5/PGRL1-dependent and NDH-dependent pathways contribute to *pmf* formation. Recently, in *Chlamydomonas*, the relative maximal capacities of two PSI cyclic transport pathways (one depending on PGRL1 and the other on type II NDH) were estimated and the major contribution of the PGRL1 pathway under reducing conditions was concluded [36].

4.2. Regulation of H^+ conductivity via ATP synthase in the pgr5 mutant

Alongside the drastic decrease in *pmf*, g_H^+ in the *pgr5* mutant was significantly greater than that in the WT, as reported previously [27]. We do not eliminate the possibility that the decay kinetics is partly affected

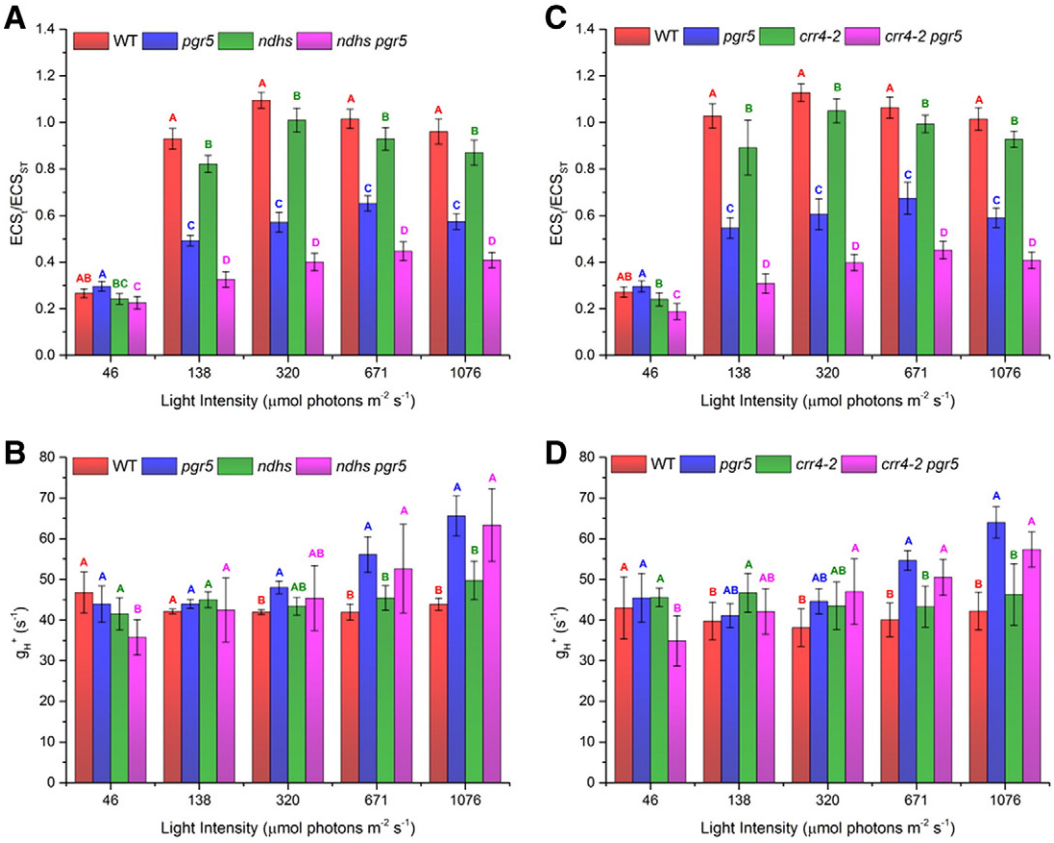

Fig. 4. ECS analysis of the WT, the *pgr5* mutant, mutants defective in NDH (*ndhs* and *crr4-2*), and their double mutants (*ndhs pgr5* and *crr4-2 pgr5*). *ndhs* was used in (A) and (B), whereas *ndh4-2* was used in (C) and (D). For each AL intensity, a 1-s dark pulse was applied three times (technical replicates) and the averages were calculated. ECS_t levels were standardized against the 515-nm absorbance change induced by a single turnover flash (ECS_{ST}). g_H^+ was estimated from a decay kinetic of the ECS signal on an abrupt light-dark transition. Data represent means ± SD ($n = 3$–7 biological replicates with 3 technical replicates). Different letters on error bars represent statistical difference ($p < 0.05$) by ANOVA.

by factors other than proton conductivity of ATP synthase. The most likely candidate is residual turnover of the Cyt b_6f complex during the dark pulse, which continues until the high potential chain is fully reduced. In the *pgr5* mutant, the high potential chain is more reduced by electrons than that in the WT, which may result in the rapid cease of *pmf* formation in the dark. In the WT, however, the size of g_H^+ did not increase at high light intensities, in which the high potential chain is more reduced by electrons (Fig. 1B), suggesting that the contribution of turnover of the Cyt b_6f complex to ECS decay in the dark is minor. The ECS analysis monitors ATP synthase activity indirectly. Despite this technical limitation, we consider that the g_H^+ parameter can be used to roughly monitor ATP synthase activity in vivo.

We discovered that the elevated levels of g_H^+ were evident at high light intensities (320 μmol photons m^{-2} s^{-1} or more) (Fig. 1B) and that the high g_H^+ levels were reversed to low-light (138 μmol photons m^{-2} s^{-1}) levels after dark incubation for 20 min (Fig. 2B). Because the same trend, but with lower amplitude, was observed in the WT, we hypothesize that the mechanism of g_H^+ regulation is the same in the *pgr5* mutant and the WT. Several mechanisms for the regulation of ATP synthase activity in chloroplasts have been elucidated [37]. Binding of ADP-Mg to the catalytic site strongly inhibits ATP hydrolysis [38]. Monitoring of the membrane potential formed across the thylakoid membrane has revealed that conformational change in the ε subunit is induced in the dark and negatively regulates ATP synthase activity [39,40]. These on–off switches likely prevent futile ATP hydrolysis in the dark. We cannot eliminate the possibility that these forms of regulation are disturbed in the *pgr5* mutant, resulting in higher g_H^+ than in the WT during prolonged dark adaptation (Fig. 2B). However, these mechanisms are unlikely to explain the elevation in g_H^+ at high light intensities.

Chloroplast ATP synthase is a thiol enzyme, the activity of which is regulated via a disulfide bond present in the CF_1 γ subunit [37]. To activate ATPase in the light, the disulfide bond is reduced by thioredoxin-*f*, which is in turn reduced by ferredoxin–thioredoxin reductase. On the basis of an unusual reduction in $P700^+$ levels [18], most likely the electron acceptors from PSI are highly reduced in the *pgr5* mutant. If this is the case, then the γ subunit would be more easily reduced in the light in the *pgr5* mutant. This idea is supported by the fact that the activity of another thiol enzyme, NADP-malate dehydrogenase (NADP-MDH), is higher at low light intensity in the *pgr5* mutant than in the WT [41]. However, the reduction of the disulfide bond in the γ subunit is more sensitive to light than is the case in other thiol enzymes, including FBPase [42]. The disulfide bond in the γ subunit is fully reduced in a few seconds at very low light intensity (4 μmol photons m^{-2} s^{-1}) [43]. Rapid activation of ATP synthase may be related, at least partly, to the thiol regulation of the γ subunit during the induction of photosynthesis. However, the lack of this regulation had relatively small effects on the maximum activity of ATP synthase [44]. Thiol regulation of the γ subunit is unlikely to be a main reason for the elevated g_H^+ at high light intensities.

After long dark adaptation (more than 2 h), g_H^+ was still higher in the *pgr5* mutant than that in the WT (Fig. 2B). On the basis of the relatively fast recovery of elevated g_H^+ caused by high light (20 min), this phenotype in g_H^+ observed after long dark adaptation may reflect a different physiological event. A candidate is inactivation of the Calvin cycle enzymes, which shows a slower response to light than that of ATP synthase [42]. In the *pgr5* mutant, however, g_H^+ was higher even after 15 to 16-min AL exposure, in which the Calvin cycle enzymes are activated to the steady-state levels (Fig. 2C). The *pgr5* mutant may accumulate

unusual forms of reducing power, which induces rapid activation of ATP synthase after long adaptation.

Although the exact molecular mechanism is unknown, the activity of ATP synthase is fine-tuned during steady-state photosynthesis in response to metabolic demand. The process has been well characterized in the response of g_H^+ to changes in CO_2 concentration [15]. The *Arabidopsis mothra* mutant, in which the γ subunit is fixed in the oxidized state, still shows modification of g_H^+ in response to changes in CO_2 concentration, suggesting that fine-tuning of ATP synthase activity is independent of the thiol modification [44]. Under low CO_2 conditions linear electron transport is slowed down, reducing the rate of ΔpH formation; under these circumstances, PGR5/PGRL1-dependent PSI cyclic electron transport is necessary to sustain the low lumen pH for qE induction [18]. Concomitantly, the g_H^+ level sharply responds to the low CO_2, helping markedly to maintain qE induction [15]. Low CO_2 levels limit the Calvin cycle, resulting in slower consumption of ATP, with which ATP synthesis has to keep in step. Although the molecular mechanism is unclear, fine-tuning of ATP synthase activity operates during steady-state photosynthesis. How is this regulation related to the g_H^+ phenotype observed in the *pgr5* mutant at high light intensities? Kanazawa and Kramer [45] and Avenson et al. [27] speculated that the fine-tuning mechanism might operate by monitoring metabolites that reflect ATP synthesis, such as [Pi]. Although the experimental evidence is still lacking, ATP levels are likely to be low in the stroma of the *pgr5* mutant, and this may activate ATP synthase. Although the reduced ATP level may be somewhat compensated for by this mechanism in the *pgr5* mutant, it seriously disturbs the regulation of photosynthetic electron transport via low lumen pH, qE induction, and downregulation of the cytochrome b_6f complex.

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Disclosures

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.11.013.

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