Does the Arabidopsis proton gradient regulation Mutant Leak Protons from the Thylakoid Membrane?1[OPEN]

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Despite generating an obvious mutant phenotype, whether the Arabidopsis (Arabidopsis thaliana) proton gradient regulation (pgr5) mutation influences cyclic electron transport (CET) around PSI is a topic of debate. Results of electrochromic shift analysis show that proton conductivity across the thylakoid membrane ($g_{H^+}$) in the pgr5 mutant is enhanced at high light intensity. Given this observation, PGR5 was proposed to regulate ATP synthase activity rather than mediating CET. The originally reported pgr5 phenotype reflects a smaller proton motive force (pmf) and could be explained by this H$^+$ leakage model. In this study, we genetically reexamined the high-$g_{H^+}$ phenotype of the pgr5 mutant. Transgenic lines in which flavodihion protein-dependent pseudo-CET replaced PGR5-dependent CET had wild-type levels of $g_{H^+}$, suggesting that the high-$g_{H^+}$ phenotype in pgr5 plants is caused secondarily by the low pmf. The pgr1 mutant shows a similar reduction in pmf because of enhanced sensitivity of its cytochrome $b_{6}f$ complex to lumenal acidification. In contrast to the pgr5 mutant, $g_{H^+}$ was lower in the pgr1 mutant than in the wild type. In the pgr1 pgr5 double mutants, $g_{H^+}$ was intermediate to $g_{H^+}$ values of the respective single mutants. It is unlikely that $g_{H^+}$ is upregulated simply in response to a low pmf. We did not observe uncoupling of the thylakoid membrane in the pgr5 mutant upon monitoring the quenching of 9-aminoacridine fluorescence. We conclude that the $g_{H^+}$ parameter may be influenced by other factors not related to the H$^+$ leakage through ATP synthase. It is unlikely that the pgr5 mutant leaks protons from the thylakoid membrane.

The light reactions of photosynthesis are driven by two photosystems, PSII and PSI, which function in the order given to mediate electron transport from water to NADP$^+$. This linear electron transport does not satisfy the ATP/NADPH production ratio required by the Calvin-Benson cycle (Allen, 2002). The requirement for additional ATP is satisfied by cyclic electron transport (CET) around PSI (Yamori and Shikanai, 2016). In angiosperms, PSI CET consists of at least two pathways (Fig. 1). The main route of electron transport is sensitive to antimycin A and depends on PROTON GRADIENT REGULATION5 (PGR5) and PGR5-LIKE PHOTO-SYNTHETIC PHENOTYPE1 (PGRL1; Munekage et al., 2002; DalCorso et al., 2008). The minor pathway is mediated by the NADH dehydrogenase-like (NDH) complex (Peltier et al., 2016). The mutants defective in both pathways showed severe mutant phenotypes in photosynthesis and plant growth (Munekage et al., 2004).

The Arabidopsis (Arabidopsis thaliana) pgr5 mutant was isolated on the basis of its high chlorophyll fluorescence phenotype at high light intensity (Munekage et al., 2002). Because of the reduced size of ΔpH across the thylakoid membrane, the mutant cannot induce the energy-dependent (qE) component of non-photochemical quenching of chlorophyll fluorescence. In addition to qE induction, lumenal acidification also downregulates electron transport through the Cyt $b_{6}f$ complex (Fig. 1). This regulatory process, called photosynthetic control, is important for protecting PSI from photodamage, especially in fluctuating light intensities (Suorsa et al., 2012; Kono et al., 2014). The pgr5 mutant cannot exert photosynthetic control due to the reduced size of ΔpH, and consequently PSI is hypersensitive to fluctuating light (Yamamoto and Shikanai, 2019).

Mainly on the basis of the reduced activity of the ferredoxin-dependent plastoquinone reduction in ruptured chloroplasts, we proposed that the Arabidopsis pgr5 mutant was defective in PSI CET (Munekage et al., 2002). A similar phenotype, though less severe, was observed in Arabidopsis mutants defective in the chloroplast NDH complex, and the activity was completely absent in double mutants defective in both pathways (Munekage et al., 2004). PGRL1 was discovered in the Arabidopsis mutant with a photosynthetic phenotype similar to the pgr5 mutant, and it is essential for the accumulation of PGR5 (DalCorso et al., 2008). PGR5 and PGRL1 proteins were discovered in the CET supercomplex including the Cyt $b_{6}f$ complex, PSI, and...
ferredoxin-dependent NADP+ reductase (FNR) in *Chlamydomonas reinhardtii* (Iwai et al., 2010; Johnson et al., 2014). Because a single amino acid alteration in PGR5 confers photosynthesis resistance to antimycin A in Arabidopsis (Sugimoto et al., 2013), and PGRL1-dependent quinone reduction is sensitive to antimycin A in vitro (Hertle et al., 2013), we proposed that the PGR5/PGRL1 pathway corresponds to the cyclic phosphorylation discovered by Arnon et al. (1954).

PGR5 is a small protein without any known motif, and it is still unclear how it is involved in PSI CET. Despite the strong mutant phenotype, it remains a topic of debate whether PGR5 is involved in PSI CET. The problem is related to lack of a definitive method for monitoring the operation of PSI CET, especially in vivo (Johnson, 2005). It was proposed that PSI CET is still operant in the *pgr5* mutant and that PGR5 is a regulator of PSI CET (Nandha et al., 2007). In contrast, Kou et al. (2015) reported that antimycin A-sensitive CET was almost completely abolished in the *pgr5* mutant in steady-state photosynthesis. One of the mysterious phenotypes of the Arabidopsis *pgr5* mutant is the enhanced proton (H+) conductivity of the thylakoid membrane (Avenson et al., 2005; Wang et al., 2015). It was monitored as the $g_{H^+}$ parameter of electrochromic shift (ECS) analysis (Kanazawa and Kramer, 2002). The $g_{H^+}$ parameter is calculated as the inverse of the time constant of ECS decay from a first-order exponential fit. A similar phenotype has also been observed in rice (*Oryza sativa*) PGR5 knockdown lines (Nishikawa et al., 2012). In the *pgr5* mutant, H+ leakage from the thylakoid membrane may be upregulated and PGR5 may be involved in the regulation of ATP synthase activity. The model for direct interaction of PGR5 with ATP synthase was proposed by Rantala et al. (2020). Because the originally reported *pgr5* mutant phenotype depends on the reduced size of ΔpH, it can be explained by this idea.

In fact, unusual H+ leakage from the thylakoid membrane in overexpressers of the mutant version of the putative H+/K+ antiporter K+ efflux antiporter3 (KEA3) mimicked the *pgr5* mutant phenotype (Wang and Shikanai, 2019). In this study, we genetically reexamined the high-$g_{H^+}$ phenotype of the *pgr5* mutant.

**RESULTS**

The *pgr5* Mutation Is Not Directly Linked to H+ Leakage from the Thylakoid Membrane

The *pgr5* mutant is defective in PSI CET, and the high $g_{H^+}$ may be secondarily caused by the reduced size of the proton motive force (pmf). If this hypothesis were true, artificial remodeling of the pmf generation system may restore $g_{H^+}$ to the wild-type level in the *pgr5* mutant background. To test this possibility, we selected Flv-dependent pseudo-CET (Fig. 1). Because Flv is not conserved in angiosperms, including Arabidopsis, we cloned the *Flo* genes from *Physcomitrella patens* and introduced them into Arabidopsis wild-type and *pgr5-1* mutant plants (Yamamoto et al., 2016). Because we also used a weak allele of *pgr5-2* in this study, we call the original strong allele *pgr5-1* (Yamamoto and Shikanai, 2019). Flv reduces oxygen to water, probably by accepting electrons from NADPH or ferredoxin (Vincente et al., 2002). Consequently, Flv-dependent pseudo-CET from water to water generates pmf without any net accumulation of NADPH, like PGR5-dependent PSI CET.

In ECS analysis, the parameter ECSt represents the light-dark difference in $A_{515}$ (ECS signal), which corresponds to the total size of pmf formed under illumination (Bailleul et al., 2010). The pmf consists of ΔpH and membrane potential (Δψ) formed across the thylakoid membrane (Shikanai and Yamamoto, 2017). Although both components contribute to pmf, only ΔpH downregulates electron transport by inducing qE and photosynthetic control. The ECSt level was standardized by the ECS signal by a single turnover flash (ECSt). In wild-type plants, the size of pmf was saturated at 252 μmol photons m$^{-2}$ s$^{-1}$ (Fig. 2A). In the *pgr5* mutant, pmf was saturated at a lower light intensity at a lower level. As reported previously, introduction of *FloA/FloB* restored the pmf to the wild-type level in the *pgr5* mutant, indicating that the contribution to pmf formation was from Flv-dependent pseudo-CET rather
wild-type background. Even under high light intensity in the pgr5-1 mutant background, $g_{H^+}$ was maintained at the wild-type level because of the active operation of Flv-dependent pseudo-CET under high light intensity (Yamamoto et al., 2016). This result suggests that PGR5 function is not directly related to regulation of $H^+$ leakage through ATP synthase. Instead, it is likely that the pgr5 mutation indirectly affects the $g_{H^+}$ parameter, possibly via the reduced size of pmf.

The pgr1 Mutation in the Cyt bfi Complex Alleviates the $g_{H^+}$ Phenotype in the pgr5 Mutant Background

If the ATP level limited the rate of the Calvin-Benson cycle in the pgr5 mutant, it would be reasonable to conclude that ATP synthase activity was enhanced to supplement ATP production. PGR5 is unlikely to regulate ATP synthase directly, but the pgr5 mutant $g_{H^+}$ phenotype may reflect the general plant response to reduced pmf. To test this possibility, we analyzed the pgr1 mutant, in which induction of nonphotochemical quenching was severely impaired due to reduced $\Delta pH$ formation, as in the pgr5 mutant (Munekage et al., 2001). The pgr1 mutant has a single amino acid alteration in the Rieske subunit of the Cyt bfi complex. This missense mutation does not affect the stability of the Rieske subunit, but it makes the Cyt bfi complex hypersensitive to lumenal acidification (Fig. 1; Jahns et al., 2002). Even in wild-type plants, the Cyt bfi complex restricts the rate of electron transport when the thylakoid lumen is acidified. This photosynthetic control is essential for protecting PSI, especially under conditions of fluctuating light intensity (Allahverdiyeva et al., 2015). In the pgr1 mutant, photosynthetic control downregulates electron transport through the Cyt bfi complex even when the thylakoid lumen is neutral at relatively low light intensity.

Figure 3A shows the light intensity dependence of pmf. In this analysis, we also characterized a weak allele of pgr5-2 in which PGR5-dependent PSI CET was partially impaired (Yamamoto and Shikanai, 2019). Consistent with a previous report (Nakano et al., 2019), the size of pmf in the pgr5-2 mutant was between those of the wild type and the pgr5-1 mutant (Fig. 3A). In the pgr1 single mutant, the size of pmf was more severely affected by the strong downregulation of electron transport at the Cyt bfi complex, and it was not reduced further in the pgr1 pgr5-1 or pgr1 pgr5-2 double mutants. Consistent with the data in Figure 2B, $g_{H^+}$ was enhanced in the pgr5-1 mutant at high light intensities. A similar trend was observed in the pgr5-2 mutant, though to a lesser degree. Unexpectedly, the size of $g_{H^+}$ was more reduced in the pgr1 mutant than in the wild-type plants (Fig. 3B). The high $g_{H^+}$ was not simply related to the reduced size of pmf. In the double mutants with two alleles of pgr5, the size of $g_{H^+}$ was between those of the pgr1 and pgr5 mutant alleles. Consequently, $g_{H^+}$ in these mutants was similar to the wild-type level, especially at 663 $\mu$mol photons m$^{-2}$ s$^{-1}$ (Fig. 3B). To explain all these results, it is probably necessary to
Figure 3. Enhanced photosynthetic control induced by the pgr1 mutation suppresses the high-\(g_{\text{H}^+}\) phenotype of the pgr5 mutants. The light intensity dependence of pmf formation (A) and \(g_{\text{H}^+}\) (B) was monitored in the wild type (WT), pgr1, mutants of the pgr5 alleles, and pgr1 pgr5 double mutants (mean ± so of \(n = 8–10\) biological replicates). Lowercase letters indicate significant differences between genotypes at 252 and 663 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) by Tukey-Kramer test (\(P < 0.05\)). PFD, Photon flux density.

Consider the different reasons for enhanced and reduced \(g_{\text{H}^+}\) in the pgr5 and pgr1 mutants, respectively (see “Discussion”).

Relaxation of \(\Delta p\text{H}\) Is Unaffected in the Ruptured Chloroplasts Isolated from the pgr5-1 Mutant

Because our assay depended solely on the ECS signal, we also analyzed the formation of \(\Delta p\text{H}\) in the light and its relaxation in the dark by monitoring the quenching of 9-aminoacridine (9-AA) fluorescence in ruptured chloroplasts (Fig. 4). In the assay, \(\Delta p\text{H}\) was formed solely depending on linear electron transport, because 100 \(\mu\)M methyl viologen was applied as a terminal electron acceptor. Figure 4A shows representative traces of quenching in ruptured chloroplasts isolated from wild-type and pgr5-1 plants. At the onset of actinic light, quenching of the 9-AA fluorescence was observed, which reflected acidification of the thylakoid lumen. The dark-light differences in the amplitude of quenching represented the size of the \(\Delta p\text{H}\) formed in the light and rose upon an increase in light intensity to 661 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\), where the quenching levels were saturated (Fig. 4B). The size of \(\Delta p\text{H}\) formed in the light did not differ between the wild-type and pgr5-1 plants at any light intensity. To monitor \(H^+\) leakage from the thylakoid lumen via ATP synthase, we monitored the recovery kinetics of 9-AA fluorescence in the dark in the presence of ADP. We did not detect any difference in the \(t_{1/2}\) value of relaxation between the wild-type and pgr5-1 plants (Fig. 4C). We did not observe any uncoupling of the thylakoid membrane in the pgr5-1 mutant.

DISCUSSION

Why is the \(g_{\text{H}^+}\) parameter enhanced in pgr5 mutants? The \(g_{\text{H}^+}\) was restored to the wild-type level by introduction of Flv into the pgr5 mutant background (Fig. 2B). If PGR5 directly involved the downregulation of ATP synthase activity and \(H^+\) was more rapidly leaked through ATP synthase in the pgr5 mutant than in the wild type, how did the artificial Flv system complement the regulatory function of PGR5? The 9-AA quenching analysis also did not suggest any uncoupling of the thylakoid membrane in ruptured chloroplasts (Fig. 4). It is unlikely that PGR5 directly interacts with ATP synthase to downregulate the activity (\(H^+\) conductivity). The idea is consistent with several lines of evidence: (1) PGR5 was detected in the supercomplex for PSI CET in C. reinhardtii (Iwai et al., 2010; Johnson et al., 2014); (2) PGR5 and PGRL1 interact with PSI in Arabidopsis (DalCorso et al., 2008); (3) ferredoxin-dependent pmf formation and ATP synthesis were impaired in ruptured chloroplasts isolated from the pgr5 mutant plants (Wang et al., 2018); and (4) the variegated leaf phenotype of the immutans mutant was alleviated by the pgr5 mutation, suggesting collaboration between PGR5 and plastid terminal oxidase to maintain the correct redox state of the plastoquinone pool during early chloroplast development (Okegawa et al., 2010). The Flv-dependent pseudo-CET likely adjusted \(g_{\text{H}^+}\) to the wild-type level by enhancing the pmf level. \(Y(\text{I})/Y(\text{II})\), which represents the ratio of PSI yield to PSII yield, remained at \(~1\) in the Flv-accumulating pgr5 mutant, reflecting the absence of CET in the pgr5 mutant background (Yamamoto et al., 2016). However, \(Y(\text{I})\) was double \(Y(\text{II})\) in the wild-type plants, suggesting
the efficient operation of PSI CET. To optimize the pmf level, PGR5 depends solely on PSI (CET), resulting in a Y(I) that is larger than Y(II), but Flv requires both photosystems (pseudo-CET).

The high-$g_{\text{H}^+}$ phenotype of the pgr5 mutants may reflect the general response of ATP synthase to the reduced size of pmf. However, $g_{\text{H}^+}$ was smaller in the pgr1 mutant than in the wild type (Fig. 3B). The high $g_{\text{H}^+}$ in pgr5 mutants is not explained simply by the response to low pmf. It is possible that $g_{\text{H}^+}$ was reduced in pgr1 because of the lower pmf size in this mutant. However, $g_{\text{H}^+}$ tended to be constant in the wild-type plants, although the size of pmf depended on the light intensity being low (33 $\mu$mol photons m$^{-2}$ s$^{-1}$) to moderate (100–252 $\mu$mol photons m$^{-2}$ s$^{-1}$; Fig. 3). In the transgenic plants accumulating the mutant version (dpgr type) of KEA3, $H^+$ was unusually leaked from the thylakoid membrane, resulting in reduced plant growth (Wang and Shikanai, 2019). It is probably necessary to maintain a certain level of pmf in chloroplasts, and it would be dangerous to drive ATP synthase more when the size of pmf is low.

How can we explain the discrepancy in $g_{\text{H}^+}$ observed in the pgr1 and pgr5 mutants? The question is related to what $g_{\text{H}^+}$ indicates. The steady-state ECS signal peaking at 515 nm is contaminated with an $A_{505}$ caused by the synthesis of zeaxanthin and also by a Q-E-related 535-nm change (Johnson and Ruban, 2014). We have to be careful in evaluating the steady-state ECS parameters. We focused on the rapid decay kinetics of the ECS signal and observed the opposite response of $g_{\text{H}^+}$ between the pgr1 and pgr5 mutants, both of which are defective in qE induction (Fig. 3). Most probably, $g_{\text{H}^+}$ mainly reflects the $H^+$ conductivity of ATP synthase during steady-state photosynthesis (Kanazawa and Kramer, 2002). This idea is supported by an early study (Schönfeld and Neumann, 1977). However, we observed that overaccumulation of the dpgr-type KEA3 also contributed to increased $g_{\text{H}^+}$ (Wang and Shikanai, 2019). Ion movement across the thylakoid membrane contributes to $g_{\text{H}^+}$, although the extent of its contribution depends on the conditions. In addition to the movement of $H^+$ or ions, charge separation in both photosystems also induces the ECS signal. The postillumination electron flux from plastocyanin (PC) to P700$^+$ is used to estimate the rate of electron flow before cessation of illumination (Fan et al., 2016) and may affect the ECS decay. At high light intensities, the Y(ND) parameter of 700 analysis representing the donor-site regulation of PSI was extremely low in the pgr5 mutant, but it was higher in the pgr1 mutant relative to the wild type (Yamamoto and Shikanai, 2019). This result suggests that the PC pool is more reduced in the pgr5 mutant and more oxidized in the pgr1 mutant relative to the wild type. We are unsure whether the fast electron transport from PC to P700$^+$ affects the ECS decay. The Q cycle in the Cyt $b_{59}$ complex also continues until all the reducing equivalents available for plastoquinone.
reduction are consumed, and this is coupled with H⁺ translocation across the thylakoid membrane. Notably, the downregulation of the Q cycle is more sensitive to luminal acidification, and the plastoquinone pool is more reduced in the pgr1 mutant (Munekage et al., 2001; Jahns et al., 2002). The Q cycle may operate for a longer period in the dark in the pgr1 mutant, contributing to the lower gH⁺. In the pgr5 mutant, however, the Q cycle may operate more efficiently in the dark because of the higher luminal pH relative to the wild type and the accumulation of Flv. Alternatively, absence of a main route for electron donation from ferredoxin to plastoquinone (PGR5-dependent PSI CET) may limit the electron pool consumed by the Q cycle in the dark. The Q cycle also may be restricted by the reduction of the PC pool because of the severe acceptor limitation from PSI in the pgr5 mutant (Takagi and Miyake, 2018).

Any hypothesis must be consistent with the fact that the size of gH⁺ in the pgr1 pgr5-1 and pgr1 pgr5-2 double mutants was between those of the single mutants. We are still unsure precisely why gH⁺ was enhanced in the pgr5 mutant. Clearly, more research is needed to characterize the impact of the Q cycle on gH⁺. However, it is necessary to amend the H⁺ leakage model of the pgr5 mutant simply depending on its high gH⁺ phenotype.

**MATERIALS AND METHODS**

### Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*; accession Columbia g1) wild type, mutants, and transgenic plants accumulating *Phycocyaninella patens* Flvs in their chloroplasts were grown in soil in a growth chamber (50 μmol photons m⁻² s⁻¹, a 9-h photoperiod, 23°C, and 55% humidity) for 6 to 8 weeks after germination. Fully expanded leaves were used for the experiments.

### In Vivo Measurements of the ECS Signal

The ECS signal was monitored as an absorption change at 515 to 550 nm using a Dual-PAM 100 equipped with a P515/535 module (Walz). Detached leaves of plants adapted to the dark for 30 min were used for the analysis. The ECS signal was detected after 3-min illumination at different actinic light intensities (33, 99, 233, 365, 700 μmol photons m⁻² s⁻¹) from 420 to 580 nm. The ruptured chloroplasts were illuminated using four consecutive cycles of 2-min illumination (96, 266, 661, and 1,143 μmol photons m⁻² s⁻¹) followed by 2-min recovery in the dark. The 9-AA fluorescence traces were normalized to the initial dark fluorescence levels at time 0.

The baseline drift of fluorescence was observed during the measurement, as shown in Figure 4A. After the baseline correction, 9-AA quenching upon actinic light illumination and recovery kinetics upon turning off actinic light was calculated. The rate constant (k) for the dark recovery of 9-AA quenching was estimated by fitting the first 1-min recovery curve with first-order exponential rise kinetics. The half-life (t₁/₂) of the 9-AA fluorescence recovery was calculated as ln2/k.

### Measurement of 9-AA Fluorescence in Ruptured Chloroplasts

Intact chloroplasts were prepared by homogenizing fresh leaves in ice cold medium (330 mM sorbitol, 5 mM MgCl₂, 10 mM NaPi₂O₆, 20 mM d-ribose, and 2.5 mM EDTA [pH 6.5]) with a polotron. The homogenate was filtered through two layers of Miracloth (Millipore). The filtrate was centrifuged for 3 min at 4,200g. The chloroplast-enriched pellet was resuspended in a small volume of resuspension medium (330 mM sorbitol, 10 mM KCl, 5 mM MgCl₂, 2.5 mM EDTA, and 50 mM HEPES [pH 7.6]) and kept on ice until use.

The measurement of 9-AA fluorescence was performed using the Dual-ENADPH and Dual-DNADPH modules (Walz) for the Dual-PAM 100. Isolated chloroplasts with 35 μg chlorophyll were added to 700 μL buffer (7 mM MgCl₂, 30 mM KCl, 2 mM KH₂PO₄, and 50 mM HEPES-KOH [pH 8.0]) in a reaction cuvette. After a 1-min incubation to osmotically burst chloroplasts, 700 μL double concentrated reaction buffer (14 mM MgCl₂, 60 mM KCl, 4 mM KH₂PO₄, 660 mM sorbitol, 100 mM HEPES-KOH [pH 8.0]), 100 μM methyl viologen, 2 mM ADP, 105 units of superoxide dismutase, and 385 units of catalase were added to the reaction cuvette. Finally, 1.8 μM 9-AA was added to the reaction and the 9-AA fluorescence was monitored. Excitation was provided by 365 nm LEDs and fluorescence emission was detected between 420 and 580 nm. The ruptured chloroplasts were illuminated using four consecutive cycles of 2-min illumination (96, 266, 661, and 1,143 μmol photons m⁻² s⁻¹) followed by 2-min recovery in the dark. The 9-AA fluorescence traces were normalized to the initial dark fluorescence levels at time 0.

### Accession Numbers

The sequence data from this article can be found in The Arabidopsis Information Resource database (https://www.arabidopsis.org/) under the following accession numbers: At2g05620 (PGR5) and At4g03280 (PGR1/petC).

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