Cyclic electron transport around photosystem I contributes to photosynthetic
induction with Thioredoxin f

Short title: The function of PGR5 in photosynthetic induction

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Y.O., L.B., and T.S. designed research; Y.O. and L.B. performed experiments; Y.O., L.B., T.S., and K.M. analyzed data; and Y.O., L.B., T.S., and K.M wrote the paper.

One-sentence summary

Cyclic electron transport around photosystem I contributes to the induction of photosynthesis by preventing overreduction of the stroma.
Abstract

In response to light, plants efficiently induce photosynthesis. Light activation of thiol enzymes by the thioredoxin (Trx) systems and cyclic electron transport by the PROTON GRADIENT REGULATION 5 (PGR5)-dependent pathway contribute substantially to regulation of photosynthesis. Arabidopsis thaliana mutants lacking f-type Trxs (trx f1f2) show delayed activation of carbon assimilation due to impaired photoreduction of Calvin-Benson cycle enzymes. To further study regulatory mechanisms that contribute to efficiency during the induction of photosynthesis, we analyzed the contributions of PSI donor- and acceptor-side regulation in the trx f1f2 mutant background. The cytochrome b6f complex is involved in PSI donor-side regulation, whereas PGR5-dependent PSI cyclic electron transport is required for both donor and acceptor functions. Introduction of the pgr1 mutation, which is conditionally defective in cytochrome b6f complex activity, into the trx f1f2 mutant background did not further affect the induction of photosynthesis, but the combined deficiency of Trx f and PGR5 severely impaired photosynthesis and suppressed plant growth under long-day conditions. In the pgr5 trx f1f2 mutant, the acceptor-side of PSI was almost completely reduced, and quantum yields of PSII and PSI hardly increased during the induction of photosynthesis. We also compared the photoreduction of thiol enzymes between the trx f1f2 and pgr5 trx f1f2 mutants. The pgr5 mutation did not result in further impaired photoreduction of Calvin-Benson cycle enzymes or ATP synthase in the trx f1f2 mutant background. These results indicated that acceptor-side limitations in the pgr5 trx f1f2 mutant suppress photosynthesis initiation, suggesting that PGR5 is required for efficient photosynthesis induction.
Photosynthesis consists of a series of electron transport reactions in the thylakoid membrane and carbon fixation reactions in the stroma. In the thylakoid reactions, electrons excised from water in Photosystem II (PSII) are transferred to NADP\(^+\) through the cytochrome \(b_{6}f\) complex and Photosystem I (PSI), resulting in the production of NADPH. Electron transport is coupled with the translocation of protons across the thylakoid membrane from the stroma to the lumen. The resulting proton motive force (pmf) is utilized in ATP synthesis. NADPH and ATP are used to fix inorganic carbon in the Calvin-Benson cycle. In addition to this linear electron transport from water to NADP\(^+\), PSI cyclic electron transport contributes to the supply of ATP for carbon fixation. PSI cyclic electron transport consists of two partially redundant pathways, namely the PROTON GRADIENT REGULATION 5 (PGR5)-dependent and NADH dehydrogenase-like (NDH) complex-dependent pathways (Munekage et al., 2002, 2004; DalCorso et al., 2008). In *Arabidopsis thaliana*, the PGR5-dependent pathway is the main route for PSI cyclic electron transport and contributes to the generation of pmf across the thylakoid membrane and the resulting ATP synthesis (Munekage et al., 2002; DalCorso et al., 2008; Wang et al., 2015).

To optimize photosynthetic reactions, chloroplasts have various regulatory mechanisms (Tikhonov, 2015). The downregulation of the cytochrome \(b_{6}f\) complex, termed photosynthetic control, is a fundamental mechanism involved in the regulation of photosynthesis (Tikhonov, 2013). To avoid acceptor-side limitation of PSI, electron transport via the cytochrome \(b_{6}f\) complex is slowed through acidification of the thylakoid lumen (Stiehl and Witt, 1969). The *Arabidopsis pgr1* mutant, which has an amino acid alteration in the Rieske subunit of the cytochrome \(b_{6}f\) complex, has a decreased electron
transport rate owing to its hypersensitivity to low luminal pH (Munekage et al., 2001; Jahns et al., 2002). Furthermore, the pgr1 mutant cannot induce the thermal dissipation of the excess light energy absorbed by the PSII antennae, which is a photoprotective mechanism of PSII to avoid oxidative stress (Muller et al., 2001; Li et al., 2002). The sensitivity of the cytochrome b_{6f} complex to luminal acidification should be optimized for efficient photosynthesis and photoprotection. PSI cyclic electron transport is also important for photosynthesis and photoprotection (Munekage et al., 2004). The Arabidopsis pgr5 mutant not only fails to induce thermal dissipation but also cannot induce photosynthetic control (Suorsa et al., 2012; Yamamoto and Shikanai, 2019). Thioredoxin (Trx) systems also play a central role in the regulation of photosynthesis (Geigenberger and Fernie, 2014; Nikkanen and Rintamaki, 2019; Yoshida et al., 2019). In the chloroplast, there are two Trx systems; the first, classically known as the ferredoxin-Trx reductase/Trx system, depends on photoreduced ferredoxin (Fd) for reducing equivalents (Schurmann and Buchanan, 2008; Buchanan, 2016), whereas the second, the NADPH-Trx reductase C (NTRC) system, uses NADPH (Serrato et al., 2004; Perez-Ruiz et al., 2006). Uniquely, the chloroplast-localized NTRC consists of both reductase (NTR) and Trx domains in a polypeptide (Serrato et al., 2004). Meanwhile, classical Trxs are small molecular weight proteins (~14 kDa) that contain a conserved WC(G/P)PC motif in the redox-active site (Schurmann and Buchanan, 2008). Trxs reduce the disulfide bonds of target proteins and thereby regulate their activities. Arabidopsis chloroplasts contain 10 Trxs (f1, f2, m1, m2, m3, m4, x, y1, y2, and z) classified into 5 types (Balsera et al., 2014; Buchanan, 2016; Kang et al., 2019). Trx m is the most abundant type and accounts for approximately 69% of all Trx proteins in the chloroplast stroma (Okegawa and Motohashi, 2015). Trx m was demonstrated to be
essential for plant growth and photoprotection, as its deficiency causes growth defects (Wang et al., 2013; Okegawa and Motohashi, 2015). Trx f is another major Trx; it accounts for approximately 22% of all Trx proteins in the stroma. Initial biochemical analyses *in vitro* have shown that Trx f plays a central role in the redox regulation of enzymes in the Calvin-Benson cycle, such as fructose-1,6-bisphosphatase (FBPase) and sedoheptulose-1,7-bisphosphatase (SBPase) (Schurmann and Buchanan, 2008; Geigenberger and Fernie, 2014; Yoshida et al., 2015). However, an *Arabidopsis* Trx f-deficient mutant (termed *trx f1f2*), which lacks both Trx f1 and Trx f2, does not show any growth differences compared with the wild type under long-day conditions (Yoshida et al., 2015; Naranjo et al., 2016). In contrast, Naranjo et al. (2016) reported that the *trx f1f2* mutant does display growth inhibition under short-day conditions. They suggested that Trx f is dispensable for plant growth but is required for the efficient induction of photosynthesis.

Here, we characterized the *trx f1f2* double mutant, the *pgr1 trx f1f2* triple mutant (in which the cytochrome b6f complex is hypersensitive to luminal acidification), and the *pgr5 trx f1f2* triple mutant (in which PGR5-dependent PSI cyclic electron transport is also disturbed). In the *trx f1f2* mutant, a delay in the activation of Calvin-Benson cycle enzymes during the induction of photosynthesis caused low activity of ATP synthase. Furthermore, the *pgr5 trx f1f2* triple mutant exhibited severe growth defects, suggesting that PGR5-dependent PSI cyclic electron transport is indispensable in the *trx f1f2* mutant background. We propose that PGR5-dependent PSI cyclic electron transport also contributes to efficient photosynthetic induction.
Results

The pgr5 trx f1f2 triple mutant exhibited severe growth defects under long-day conditions

To examine the effect of the pgr1 and pgr5 mutations on photosynthesis in the trx f1f2 mutant background, the triple mutants pgr1 trx f1f2 and pgr5 trx f1f2 were generated by crossing. Whereas the pgr1 trx f1f2 plants were indistinguishable from the wild-type plants under long-day conditions, growth was severely affected in the pgr5 trx f1f2 mutant (Figure 1A and Supplemental Figure S1A). There was no difference in the fresh weight of 3-week-old plants among the wild-type, pgr1, pgr5, trx f1f2, and pgr1 trx f1f2 plants (Figure 1B). In contrast, the fresh weight of the pgr5 trx f1f2 plants was less than half of that of the wild-type plants (Figure 1B). Furthermore, the chlorophyll content in the pgr5 trx f1f2 leaves was approximately 58% of that in the wild-type leaves (Figure 1C). These results indicated that the combination of the pgr5 and trx f1f2 mutations led to severe growth defects. Interestingly, the growth retardation in the pgr5 trx f1f2 plants was less evident when they were grown under continuous light conditions (Supplemental Figure S1B). The fresh weight of the pgr5 trx f1f2 plants was approximately 74% of that of the wild-type plants (Supplemental Figure S1C), and its chlorophyll content was almost the same as that of the pgr5 mutant (Supplemental Figure S1D). Since Trx f was proposed to be a requirement for the effective induction of photosynthesis (Naranjo et al., 2016), continuous light conditions may be better for the pgr5 trx f1f2 plants.

To examine the influence of both the pgr5 and trx f1f2 mutations on the stability of photosynthesis-related proteins, western blot analysis was performed (Figures 1D and E). As reported previously (Yoshida et al., 2015), in the trx f1f2 mutant, the protein levels of other Trx isoforms did not change (Figure 1D). In the pgr5 trx f1f2 mutant, the
accumulation of Calvin-Benson cycle enzymes and subunits of the photosynthetic complexes were comparable to that in the wild type (Figures 1D and E).

The quantum yield of PSII was severely impaired in the pgr5 trx f1f2 mutant

To characterize photosynthetic activity in the mutants, their chlorophyll fluorescence parameters were analyzed using a Mini PAM II fluorometer. The maximum quantum yield of PSII ($F_v/F_m$) was lower in the pgr5 trx f1f2 mutant than in the other genotypes (Figure 2A). To assess the functionality of PSII in the pgr5 trx f1f2 mutant under growth light conditions, qPd was measured according to Ruban and Murchie (2012). qPd represents the redox state of the QA site of PSII in the dark and is used to monitor the level of photoinhibition caused by both donor- and acceptor-side limitations of PSII (Wilson and Ruban, 2019). The qPd level less than 0.98 indicates plants to be photoinhibited (Ruban and Murchie, 2012). In the pgr5 trx f1f2 mutant, qPd was lower than 0.98 after actinic light (AL) illumination (0.855 ± 0.061; Figure 2B), indicating that PSII of the pgr5 trx f1f2 mutant was photoinhibited even under constant low-light conditions, although the accumulation of PSII subunits was not affected (Figure 1E). The light-intensity dependence of the effective quantum yield of PSII [Y(II)] and non-photochemical quenching chlorophyll fluorescence (NPQ) were also measured. As reported previously (Munekage et al., 2001; Munekage et al., 2002), Y(II) was lower in both the pgr1 and pgr5 mutants than in the wild type at high light intensities (Figure 2C). The trx f1f2 mutant showed substantially decreased Y(II) at low light intensities but a similar Y(II) level to that in the wild type at light intensities higher than 100 μmol photons m$^{-2}$ s$^{-1}$ (Figure 2C). Moreover, a similar trend was also observed in the pgr1 trx f1f2 mutant; here, Y(II) was lower than that in the pgr1 mutant at low light intensities,
whereas it was identical to that in the *pgr1* mutant at light intensities higher than 200
µmol photons m$^{-2}$ s$^{-1}$. In contrast, Y(II) remained lower in the *pgr5 trx f1f2* mutant under
all light intensities, compared to that in the *pgr5* and *trx f1f2* mutants (Figure 2C).

The NPQ level mainly reflects the size of thermal dissipation in plants. The
ΔpH-dependent component of NPQ (qE) was induced in the wild type at light intensities
higher than 50 µmol photons m$^{-2}$ s$^{-1}$ (Figure 2D). In the *pgr1* and *pgr5* single mutants, the
decreased ΔpH caused a low NPQ level. In contrast, the *trx f1f2* mutant showed higher
NPQ than the wild type. In the *pgr1trx f1f2* mutant, the NPQ was slightly higher at low
light intensities than that in the *pgr1* mutant (Figure 2D). Unexpectedly, the *pgr5 trx f1f2*
mutant induced a higher NPQ, especially at low light intensities, compared to that in the
wild type, and the level was almost identical to that in the *trx f1f2* mutant (Figure 2D).

In the analysis of light-intensity dependence, the AL intensity was increased in a
step-wise manner at every 2 min after applying a saturating pulse (SP). Since Trx f has
been suggested to function in the activation of photosynthesis (Naranjo et al., 2016),
photosynthesis may not have been activated in the *trx f1f2* mutant background at low light
intensities. To evaluate this possibility, Y(II) and NPQ were assessed during the
induction of photosynthesis at a low light intensity of 75 µmol photons m$^{-2}$ s$^{-1}$ (Figures
2E and F). Y(II) reached the steady-state level within 5 min after the onset of AL in the
wild type. In the *trx f1f2* mutant, however, it took more than 10 min for Y(II) to reach a
steady-state level, but the final value was almost identical to that in the wild type (Figure
2E). The *pgr5 trx f1f2* mutant showed markedly lower Y(II) than the *pgr5* mutant and the
Y(II) did not increase at all even 20 min after the onset of AL (Figure 2E). This result was
consistent with the growth defect of the *pgr5 trx f1 f2* mutant (Figures 1A and B).
Consistent with the result of the light intensity-dependence analysis (Figure 2D), the
\textit{pgr5 trxf1f2} mutant induced a higher NPQ than the wild type (Figure 2F). The qE
component of NPQ is characterized by its relatively fast relaxation kinetics on a
physiological time scale of seconds to several minutes (Horton et al., 1996). The majority
of NPQ induced in the \textit{pgr5 trxf1f2} mutant was relaxed within several minutes in the dark
(Figure 2F). These results indicated that NPQ in the \textit{pgr5 trxf1f2} mutant was largely
dependent on the qE component, suggesting that the restoration of NPQ in the \textit{pgr5 trx
\textit{f1f2}} mutant is attributed to a concomitant restoration of $\Delta$H.

We also measured linear electron transport to NADP$^+$ in ruptured chloroplasts
(Figure 2G). Fd and NADP$^+$ were added exogenously as electron acceptors to ruptured
chloroplasts. The \textit{pgr1} mutant showed lower Y(II) than the wild type at a light intensity of
167 $\mu$mol photons m$^{-2}$ s$^{-1}$ owing to its hypersensitivity to low luminal pH (Munekage et
al., 2001; Jahns et al., 2002), whereas the \textit{pgr1 trxf1f2} mutant had the same Y(II) as the
\textit{pgr1} mutant (Figure 2G). In contrast, there was no difference in the Y(II) values among
the wild type and \textit{pgr5, trx f1f2}, and \textit{pgr5 trxf1f2} mutants (Figure 2G). These results
indicated that the PSII and PSI activities were not affected in the \textit{trx f1f2} mutant
background, suggesting that the markedly decreased Y(II) in the \textit{pgr5 trxf1f2} mutant was
caused by acceptor-side, but not donor-side, limitations of PSI.

The acceptor-side of PSI was highly reduced in the \textit{pgr5 trxf1f2} mutant even at low
light intensity

The \textit{pgr5 trxf1f2} mutant was suggested to be limited on the acceptor-side of PSI.
Therefore, we next simultaneously measured the chlorophyll fluorescence and absorption
changes in P700 using a Dual-PAM-100 system (Figure 3). Plants were dark-adapted for
30 min and then illuminated with AL (75 μmol photons m$^{-2}$ s$^{-1}$) for 5 min. The Y(I) parameter is defined by the fraction of P700 that is reduced and not limited by the acceptor side (Klughammer and Schreiber, 2008) and is often used to estimate the effective quantum yield of PSI. In the wild type, Y(I) and Y(II) rapidly increased after a shift from dark to light (Figures 3A and B). As reported previously (Naranjo et al., 2016), in the trx f1f2 mutant, the initial increases in Y(I) and Y(II) were markedly delayed compared with those in the wild type, and high NPQ was maintained over time (Figures 3A, B, and E). This was accompanied by a delay in the relaxation of the acceptor-side limitation of PSI monitored based on Y(NA) (Figure 3C). Most likely, this phenotype was due to delayed activation of Calvin-Benson cycle enzymes followed by a shortage of electron acceptors in the trx f1f2 mutant. Y(ND), the PSI donor-side limitation in electron transport, is used to estimate the operation of photosynthetic control. In the pgr1 mutant, the transient peak of Y(ND) formed within 60 s of AL onset was higher than that in the wild type owing to enhanced photosynthetic control (Figure 3D). In contrast, the trx f1f2 mutant did not form this peak; instead, a gradual rise in Y(ND) was observed, peaking at 3 min after the onset of AL. This suggested that the thylakoid lumen became acidic during this period. The introduction of the pgr1 mutation into the trx f1f2 mutant background did not substantially affect its PSII or PSI photochemistry, though NPQ was partially induced even in the pgr1 mutant background (Figure 3E). PGR5-dependent PSI cyclic electron transport is required to protect the stroma from overreduction (Munekage et al., 2002; DalCorso et al., 2008). In the pgr5 mutant, the P700$^+$ level was drastically reduced at high light intensities (Supplemental Figure S2). Even under the low light intensity used in this study, the relaxation of Y(NA) was markedly delayed in the pgr5 mutant (Figure 3C). The increase in Y(I) was also delayed, but it reached the wild-type level within 3 min of
AL onset (Figure 3A). In contrast, the increases in Y(I) and Y(II) were severely suppressed in the pgr5 trx f1f2 mutant during the induction of photosynthesis (Figures 3A and B). The high level of Y(NA) was not relaxed at all during the 5 min of illumination (Figure 3C). Consequently, Y(ND) was close to zero, indicating that the acceptor-side of PSI was largely reduced in the pgr5 trx f1f2 mutant, even under low light conditions. These results indicated that a combination of pgr5 and trx f1f2 mutations synergistically disturbed the initiation of photosynthesis.

The high level of Y(NA) in the pgr5 trx f1f2 mutant was not relaxed within 5 min of AL onset (Figure 3C). To determine whether this acceptor-side limitation could be observed during steady-state photosynthesis, PSI and PSII photosynthetic parameters were measured without dark adaptation (Supplemental Figure S3). In contrast to that in the induction phase of photosynthesis, no difference in the photosynthetic parameters was observed between the wild type and trx f1f2 mutant (Figure 3 and Supplemental Figure S3), indicating that Trx f deficiency does not affect photosynthesis under steady-state conditions. At this light intensity (75 µmol photons m$^{-2}$ s$^{-1}$), the parameters in the pgr5 mutant were almost the same as those in the wild type. In contrast, in the pgr5 trx f1f2 mutant, Y(I) was only slightly increased and high Y(NA) was only slightly relaxed, compared to those in the induction phase of photosynthesis (Figure 3 and Supplemental Figure S3). These results suggest that PGR5-dependent PSI cyclic electron transport is required to prevent overreduction of the PSI acceptor-side, especially in the trx f1f2 mutant background, even under constant low-light conditions.

The relaxation of proton motive force was delayed in the trx f1f2 mutant.
The *trx f1f2* mutant exhibited the induction of higher NPQ than the wild type (Figure 2D and Figure 3E). Furthermore, NPQ was also higher in the *pgr1 trx f1f2* and *pgr5 trx f1f2* mutants than in the *pgr1* and *pgr5* single mutants, respectively (Figure 2D and Figure 3E). To investigate the reason for this increase in NPQ in the *trx f1f2* mutant background, the electrochromic shift (ECS) was analyzed using a Dual PAM system. The ECS signal represents an absorbance change at 515 nm owing to photosynthetic pigments, which is affected by the electric field formed across the thylakoid membrane. ECS is the light-dark difference in the ECS signal, and represents the magnitude of the pmf formed in the light. ECS was standardized against ECS ST, which is the ECS signal induced by a single turnover light pulse using dark-adapted leaves. The *g* H+ parameter is determined by monitoring the decay kinetics of the ECS signal in the dark and is considered to mainly represent the proton conductivity of ATP synthase (Takizawa et al., 2008), although the careful inspection is needed for the mutants including *pgr1* and *pgr5* (Yamamoto and Shikanai, 2020). During the induction of photosynthesis, in the wild type, high-level pmf was transiently formed, decreasing to the steady-state level within 3 min of AL onset (Figure 4A). Conversely, *g* H+ increased during the induction of photosynthesis, mainly reflecting the activation of chloroplast ATP synthase (Figure 4B). This process corresponded with the induction and relaxation of NPQ (Figure 3E). The increase in *g* H+ was suppressed in the *trx f1f2* mutant compared to that in the wild type, resulting in delayed relaxation of the transiently induced pmf (Figures 4A and B). The high-NPQ phenotype observed in the *trx f1f2* mutant is probably explained by the suppression of ATP synthase activity. In the *pgr1* mutant background, the *trx f1f2* defects also enhanced pmf and lowered *g* H+ (Figures 4A and B), consequently inducing higher NPQ than that in the *pgr1* mutant (Figure 3E). In contrast, the *pgr5* mutation further decreased the *g* H+ level.
in the \textit{trx f1f2} mutant background during the period 150–270 s after the onset of AL (Figure 4B). The \textit{pgr5 trx f1f2} triple mutant did not exhibit the induction of transient NPQ (Figure 3E), and the level of pmf after 270 s of AL onset was similar to the wild-type level and lower than the level observed in the \textit{trx f1f2} mutant (Figure 4A). This is probably because of the very low level of linear electron transport (Figures 3A and B). Despite the constantly low pmf level, the \textit{pgr5 trx f1f2} mutant induced a moderate NPQ level (Figure 3E). A similar trend was also observed when the light-intensity dependence of these parameters was analyzed (Supplemental Figure S4). The \textit{pgr5 trx f1f2} mutant showed low pmf similar to that in the \textit{pgr5} mutant but induced a higher NPQ than the \textit{pgr5} mutant (Supplemental Figure S2E).

\textbf{Photoactivation of FBPase and SBPase was delayed in the \textit{trx f1f2} mutant}

In the \textit{trx f1f2} mutant, the \(g_{\text{H}^+}\) was lower than that in the wild type (Figure 4B). ATP synthase in chloroplasts is light-activated via reduction of the CF\(_{1}\)-\(\gamma\) subunit (Nalin and Mccarty, 1984). Trx \(f\) contributes to the light-dependent reduction of thiol enzymes, including ATP synthase and Calvin-Benson cycle enzymes (Schwarz et al., 1997). The lower \(g_{\text{H}^+}\) might have reflected the impaired light-dependent reduction of ATP synthase. To investigate this possibility, we examined the redox state of several thiol enzymes during the induction of photosynthesis and under constant low light conditions (80 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)). The thiol enzyme levels in the \textit{trx f1f2} mutant background were not affected (Figures 1D and E). The light-induced state changes in the CF\(_{1}\)-\(\gamma\) subunit were determined by labeling the free thiols with the thiol-reactive 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) reagent. In the wild type, CF\(_{1}\)-\(\gamma\) was rapidly reduced upon illumination from zero to the steady-state level over 300
s (Figure 5A). This occurred in all the genotypes (Figure 5A), suggesting that the lower
level of $g_{H}^{+}$ in the $trx f1f2$ mutant background was not caused by the suppressed activation
of ATP synthase due to the impaired reduction of CF$_{1}$-$\gamma$. As reported previously (Naranjo
et al., 2016), however, the light-dependent reduction of FBPase was delayed and the final
reduction level was lower in the $trx f1f2$ mutant background (Figure 5B). In the wild type,
FBPase was gradually reduced within 30 s of the onset of illumination, whereas 300 s was
required to start reducing FBPase in the $trx f1f2$ mutant background (Figure 5B). SBPase
also needs to be photoreduced to be active. Compared with that of FBPase, the reduction
of SBPase was slower even in the wild type (Figure 5C). As observed for FBPase, the
reduction of SBPase was delayed in the $trx f1f2$ mutant background, but the final
reduction level was almost identical to that in the wild type (Figure 5C). Consistent with a
previous report (Naranjo et al., 2016), these results indicated that Trx $f$ is particularly
important for the activation of Calvin-Benson cycle enzymes during the induction of
photosynthesis. The low $g_{H}^{+}$ in the $trx f1f2$ mutant was suggested to be indirectly caused
by the delayed activation of the Calvin-Benson cycle. In the $pgr1 trx f1f2$ and $pgr5 trx$
$f1f2$ triple mutants, the reduction of FBPase and SBPase was delayed, as in the $trx f1f2$
mutant, but their reduction levels were not further suppressed (Figures 5B and C). These
results suggested that the higher Y(NA) and lower Y(I) and Y(II) phenotypes observed in
the $pgr5 trx f1f2$ triple mutant were not caused by differences in the reduction of thiol
enzymes related to the $trx f1f2$ mutations.
Discussion

**PGR5-dependent PSI cyclic electron transport is required for normal plant growth in the trxF1F2 mutant background**

Here, we applied a genetic approach to evaluate the influence of PSI donor- and acceptor-side limitations on the function of Trx f. The *pgr1* and *pgr5* mutants exhibited a similar phenotype, with low ETR and NPQ values, but these mutations had distinct effects in the *trxF1F2* mutant background. The combination of the *pgr1* and *trxF1F2* mutations did not affect plant growth; instead, the *pgr1 trxF1F2* mutant showed a more rapid increase in Y(I) and relaxation of Y(NA) in the 40–100 s period after the onset of AL compared to those in the *trxF1F2* mutant (Figure 3). These results suggest that the donor-side limitation of PSI in the *pgr1* mutant partially alleviated the acceptor-side limitation of PSI in the *trxF1F2* mutant. In contrast to that with the *pgr1* mutation, the introduction of the *pgr5* mutation into the *trxF1F2* mutant background caused severe growth defects (Figure 1). In the *pgr5 trxF1F2* mutant, the values of Y(I) and Y(II) were markedly lower, and that of Y(NA) was substantially higher than the values in the *pgr5* and *trxF1F2* mutants, even under low light conditions (Figure 3 and Supplemental Figure S2). This indicated that the acceptor limitation of PSI was synergistically enhanced in the *pgr5* and *trxF1F2* mutants. The introduction of the *pgr5* mutation into the *chlororespiratory reduction 2-2 (crr2-2)* mutant background also leads to severe growth defects (Munekage et al., 2004). The *crr2-2* mutant is deficient in the NDH complex-dependent pathway owing to the lack of expression of the *ndhB* gene (Hashimoto et al., 2003). Recently, the activity of the NDH complex was proposed to be regulated by the Trx systems (Courteille et al., 2013; Nikkanen et al., 2018). Nikkanen et al. (2018) reported that NTRC interacts with NdhH, PnsB1 (Ndh48), NdhS, NdhU, and...
NdhO, which are subunits of the NDH complex, and the overexpression of NTRC enhances NDH activity. Conversely, Trx m4 was suggested to downregulate NDH activity (Courteille et al., 2013). However, in contrast to the crr2-2 pgr5 mutant, which cannot induce NPQ (Munekage et al., 2004), the pgr5 trx f1f2 mutant induced a higher NPQ (Figure 2D). Furthermore, the introduction of the crr2-2 mutation into the weak mutant allele of pgr5 further lowered pmf (Nakano et al., 2019), but the pgr5 trx f1f2 mutant showed a similar level of pmf as the pgr5 mutant (Supplemental Figure S4). These results suggest that NDH activity is not affected in the trx f1f2 mutant background and that the phenotype of the pgr5 trx f1f2 mutant is not due to a lack of NDH complex-dependent PSI cyclic electron transport, unlike that with the crr2-2 pgr5 mutant.

A study on the high cyclic electron flow 1 (hcef1) mutant, defective in FBPase, showed that the loss of FBPase activity leads to enhancement of NDH complex-dependent PSI cyclic electron transport (Livingston et al., 2010). In the hcef1 mutant, the levels of NDH subunits were enhanced and NDH activity was stimulated. Although FBPase was less active in the trx f1f2 mutant than in the wild type (Figure 5B), the level of the NDH subunit (PnsB1) did not change (Figure 1E). These results suggest the distinctly different responses of the NDH complex to the hcef1 and trx f1f2 mutations. Since the protein level of FBPase did not decrease in the trx f1f2 mutant (Figure 1D), other Trx systems could partially compensate in terms of the activation of FBPase. In fact, in the trx f1f2 mutant, the photoreduction rate of FBPase reached approximately 40% of that in the wild type during steady-state photosynthesis (Figure 5B). Trx m has also been suggested to contribute to the activation of FBPase in vivo (Okegawa and Motohashi, 2015).
Unexpectedly, the \textit{pgr}5 \textit{trx}f1f2 mutant induced a higher NPQ than the \textit{pgr}5 mutant (Figures 2D, F and Figure 3E). The \textit{trx}f1f2 mutant also exhibited higher NPQ than the wild type, especially during the induction of photosynthesis (Figure 2F and Figure 3E). In the \textit{trx}f1f2 mutant, the activation of Calvin-Benson cycle enzymes was delayed during the induction of photosynthesis, though ATP synthase was activated at the same time as that in the wild type (Figure 5). The suppression of Calvin-Benson cycle activation may indirectly lower the activity of ATP synthase, resulting in a decreased $g_{H^+}$ and an increased pmf in the \textit{trx}f1f2 mutant (Figure 4). The similar phenotype was also observed in the wild type, when the CO$_2$ concentration was lowered and the activity of the Calvin-Benson cycle was suppressed (Avenson et al., 2005). Lowering CO$_2$ causes a decrease in $g_{H^+}$, resulting in an increase in both pmf and NPQ (Avenson et al., 2005). The \textit{trx}f1f2 mutations substantially decreased $g_{H^+}$ in the \textit{pgr}5 mutant background (Figure 4 and Supplemental Figure S4). However, the \textit{pgr}5 \textit{trx}f1f2 mutant had a lower pmf level than the wild type, which was similar to that in the \textit{pgr}5 mutant, (Figure 4 and Supplemental Figure S4), probably due to markedly decreased electron transport activity (Figures 3A and B). It is still unclear why the \textit{pgr}5 \textit{trx}f1f2 mutant induced a higher NPQ than the \textit{pgr}5 mutant despite the low pmf. To explain this NPQ phenotype, we might have to consider the larger contribution of $\Delta p$H to pmf. In the \textit{pgr}5 mutant, the contribution of $\Delta p$H to pmf was reported to be larger than that in the wild type (Shikanai and Yamamoto, 2017). A slight change in the ratio of pmf components may lead to higher NPQ induction in the \textit{pgr}5 \textit{trx}f1f2 mutant. Meanwhile, it may be dangerous to absolutely rely on the ECS signals, especially in the mutants (Yamamoto and Shikanai, 2020). The steady-state ECS signal overlaps with the absorption change at 505 nm caused by zeaxanthin synthesis and the absorption change at 535 nm caused by qE induction (Johnson and Ruban, 2014).
Overall, our results indicated that donor-side limitation of PSI had no additional effect on the \textit{trx f1f2} mutation, but acceptor-side limitation of PSI enhanced the phenotypic effects of the \textit{trx f1f2} mutations. This suggests that PGR5-dependent PSI cyclic electron transport is needed for plant growth in the \textit{trx f1f2} mutant background.

\textbf{PGR5-dependent PSI cyclic electron transport is required for the induction of photosynthesis in the \textit{trx f1f2} mutant background}

The growth defects of the \textit{pgr5 trx f1f2} mutant were partially alleviated by growing the plants under continuous light conditions (Figure 1 and Supplemental Figure S1). This result is consistent with a previous report indicating that the \textit{trx f1f2} mutant shows growth inhibition under short-day, but not long-day, conditions (Naranjo et al., 2016). Since the induction of photosynthesis was delayed owing to retardation in the activation of Calvin-Benson cycle enzymes in the \textit{trx f1f2} mutant background (Figure 3 and Figure 5; Naranjo et al., 2016), a shorter day length would be deleterious for the \textit{trx f1f2} mutant background plants. During steady-state photosynthesis, the \textit{trx f1f2} mutant and wild type showed similar photosynthetic parameter levels (Supplemental Figure S3). However, in the \textit{pgr5 trx f1f2} mutant, \textit{Y(II)} remained very low also during steady-state photosynthesis, although the \textit{Y(I)} and \textit{Y(NA)} values recovered slightly, compared to those in the induction phase of photosynthesis (Figure 3 and Supplemental Figure S3). These results indicated that continuous light conditions slightly relaxed the acceptor-side limitation of PSI, resulting in the alleviation of growth defects in the \textit{pgr5 trx f1f2} mutant (Supplemental Figures S1B-E). In the \textit{pgr5 trx f1f2} mutant, the thiol enzymes were activated during steady state photosynthesis, although the reduction level of FBPase was as low as that in the \textit{trx f1f2} mutant (Figure 5). Therefore, continuous light would be
suitable for the growth of the pgr5 trx f1f2 plants. However, since PGR5-dependent PSI
cyclic electron transport is more important to protect PSI under high light conditions
(Munekage et al., 2002), the pgr5 trx f1f2 mutant may show more severe growth defects
when grown at higher light intensities, even under continuous light conditions.

The ntrc trx f1 double mutant also exhibits severe growth defects (Thormahlen et al.,
2015; Nikkanen et al., 2016). In this mutant, the light activation of FBPase and
ADP-glucose pyrophosphorylase was almost completely suppressed and the
NADPH/NADP\(^+\) ratio was increased (Thormahlen et al., 2015). NTRC has been
suggested to contribute to photosynthetic metabolism, especially under low light
conditions (Carrillo et al., 2016). As NTRC uses NADPH as an electron donor, NTRC
deficiency may enhance the reduction state of the stroma in the trx f1 mutant background.
In fact, photoinhibition of PSI was observed in the ntrc trx f1 mutant (Thormahlen et al.,
2015). Furthermore, compared to that in the wild type, the ntrc trx f1 mutant showed
increased activation of NADP-malate dehydrogenase (MDH), which reflects the stromal
redox state (Foyer et al., 1992). This indicated that the acceptor-side of PSI was limited in
the ntrc trx f1 mutant. Together with the results in the pgr5 trx f1f2 mutant, these results
suggest that the acceptor-side limitation of PSI leads to the impaired activation of
photosynthesis, resulting in plant growth defects. In the pgr5 single mutant, the induction
of photosynthesis was only delayed compared to that in the wild type, at least under the
low light conditions used in this study. However, in the trx f1f2 mutant background, the
pgr5 mutation markedly suppressed photosynthetic activity. We propose that
PGR5-dependent PSI cyclic electron transport is required to induce photosynthesis
effectively by preventing overreduction of the stroma. Since PSI cyclic electron transport
has been proposed to be regulated by the availability of electron acceptors from PSI
(Breyton et al., 2006; Okegawa et al., 2008), the function of PGR5-dependent PSI cyclic electron transport in maintaining stromal redox states may become more evident in the $trx^{f1f2}$ mutant background.
Materials and Methods

Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Columbia 0 was used as the wild type. The T-DNA insertion lines SALK_128365 (trx f1) (Thormahlen et al., 2013) and GK-020E05 (trx f2) (Yoshida et al., 2015) were obtained from the Nottingham Arabidopsis stock center (NASC, UK). To generate a double mutant, trx f1 and trx f2 T-DNA single mutants were crossed. The triple homozygous mutants, pgr1 trx f1f2 and pgr5 trx f1f2 were obtained by crossing the trx f1f2 mutant with the pgr1 or pgr5 mutant, respectively. The presence of mutations and T-DNA insertions was confirmed using PCR (for primers, see Supplemental Table S1).

Plants were grown in soil or in petri dishes containing Murashige and Skoog (MS) medium with 1.0% (w/v) agar and 1% (w/v) sucrose and grown for 3 to 5 weeks in growth chambers (50 µmol photons m$^{-2}$ s$^{-1}$, 16 h light/8 h dark cycles or continuous light, 23°C).

Analysis of chlorophyll content

Leaves (30 mg fresh weight) were harvested from 3-week-old seedlings grown on MS plates and immediately powdered by grinding in liquid nitrogen. Chlorophyll was extracted in 80% acetone (v/v) and collected by centrifugation at 15,000 × g for 5 min at 4°C. The residue was re-extracted with 80% acetone and centrifuged once again (15,000 × g, 5 min, 4°C). The chlorophyll content was determined via spectrophotometry, as described previously (Porra et al., 1989).

Isolation of chloroplasts
Chloroplasts were isolated from leaf tissue samples (1.0 g) using a Polytron PT 10-35 GT homogenizer (Kinematica, Switzerland) in 20 mM Tricine–NaOH, pH 8.4, containing 400 mM sorbitol, 5 mM MgCl$_2$, 5 mM MnCl$_2$, 2 mM EDTA, 10 mM NaHCO$_3$, 0.5% (w/v) bovine serum albumin, and 5 mM ascorbate. After centrifugation at 3,000 × g for 5 min (4°C), the pellet was gently resuspended in 50 mM HEPES–KOH, pH 7.6, containing 400 mM sorbitol, 5 mM MgCl$_2$, and 2.5 mM EDTA. Isolated intact chloroplasts were suspended in 25 mM HEPES–KOH, pH 7.6, containing 3 mM MgCl$_2$. The insoluble fraction containing thylakoids and envelopes was separated from the stroma fraction by centrifugation at 10,000 × g for 3 min at 4°C.

**SDS PAGE and western blot analysis**

Proteins were separated by SDS-PAGE, using the conventional Laemmli (Tris–glycine) system (Laemmli, 1970) or using a Tris-tricine buffer system (for PGR5 detection) (Schagger and von Jagow, 1987), and transferred onto polyvinylidene difluoride membranes. Specific antibodies against Trx-isoforms, SBPase, FBPase, NADP-MDH, CYP20-3, ATP synthase CF$_1$-ɤ (ATPC1), PGR5, and PGRL1 were prepared as described previously (Okegawa and Motohashi, 2015, 2016). For PsbA, PsbQ, PsaA, PsaF, violaxanthin de-epoxidase (VDE), zeaxanthin epoxidase (ZEP), and PsbS, commercially available polyclonal antibodies (Agrisera, Sweden) were used. Immunoblot signals were visualized using the Immobilon western chemiluminescent HRP substrate (EMD Millipore, USA) or ECL Plus Western blotting detection kit (GE Healthcare, USA). The chemiluminescence was detected using a LAS-3000UV mini lumino-image analyzer (Fujifilm, Japan).
In vitro assay of linear electron transport activity

Measurement of linear electron transport activity was performed using isolated chloroplasts, as described previously (Munekage et al., 2002). Intact chloroplasts (20 µg ml⁻¹) were osmotically ruptured in 50 mM HEPES/NaOH, pH 7.6, containing 7 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, 30 mM KCl, and 0.25 mM KH₂PO₄. Linear electron transport activity was determined based on the effective quantum yields of PSII [Y(II)] at 17 and 167 µmol photons m⁻² s⁻¹ using a Mini-PAM (pulse amplitude modulation) portable chlorophyll fluorometer (Walz, Germany). Before measurement, the electron acceptors Spinach Fd (5 µM; Sigma, USA) and NADP⁺ (1 mM; Oriental Kobo, Japan) were added.

In vivo measurements of chlorophyll fluorescence and P700 absorption changes

Chlorophyll fluorescence was measured using a Mini-PAM II (pulse-amplitude modulation) portable chlorophyll fluorometer (Walz, Germany) for the analysis depicted in Figure 2. Minimum fluorescence (Fo) was obtained from the open PSII reaction centers in the dark-adapted state, using a weak measuring light (red light, 654 nm, 0.05–0.1 µmol photons m⁻² s⁻¹). An SP of red light (800 ms, 3,000 µmol photons m⁻² s⁻¹) was applied to determine the maximum fluorescence with closed PSII centers in the dark-adapted state (Fm) and during illumination with red actinic light (AL) (Fm'). The steady-state fluorescence level (Fs) was recorded during red AL illumination. The maximum quantum yield of PSII (Fv/Fm) was calculated as (Fm − Fs)/Fm. Y(II) and NPQ were calculated as (Fm’ − Fs)/Fm' and (Fm − Fm'')/Fm', respectively (Genty et al., 1989). The value of photochemical quenching in the dark (qPd) was calculated according to the method described by Ruban and Murchie (2012) as follows: qPd = (Fm' − Fo'act)/
$(Fm' - Fo'_{calc})$, where $Fo'_{calc} = 1/(1/Fo - 1/Fm + 1/Fm')$. Far-red light (737 nm) was used to determine $Fo$ and $Fo'_{act}$. qPd was induced by illumination at 50 μmol photons m$^{-2}$ s$^{-1}$ (growth light) for 15 min. Otherwise, chlorophyll fluorescence and chlorophyll P700 absorption changes in the PSI reaction center were measured simultaneously using a portable chlorophyll fluorometer (DUAL-PAM-100 [MODULAR version] analyzer equipped with a P700 dual-wavelength emitter at 830 and 870 nm; Walz, Germany). The plants were kept in the dark for 30 min before each measurement, and detached leaves were used for the analysis. Red measuring light (620 nm) and AL (635 nm) were used for analysis. A saturating pulse of red light (300 ms, 10,000 μmol photons m$^{-2}$ s$^{-1}$) was applied to determine $Fm$ and $Fm'$.

The redox change of P700 was assessed by monitoring the absorbance changes to transmitted light at 830 and 875 nm. Pm (the level of the P700 signal of maximum oxidizable P700) was determined by the application of an SP in the presence of far-red light (720 nm). The maximal level of oxidized P700 during AL illumination ($Pm'$) was determined by SP application. The P700 signal $P$ was recorded immediately before an SP. $Y(I)$ was calculated as $(Pm' - P) / Pm$. $Y(NA)$ was calculated as $(Pm - Pm') / Pm$. $Y(ND)$ was calculated as $P / Pm$. Three complementary quantum yields were defined as follows: $Y(I) + Y(NA) + Y(ND) = 1$ (Klughammer and Schreiber, 1994). The relative level of reduced P700 was calculated as $1 - Y(ND)$. The value can vary between 0 (P700 fully oxidized) and 1 (P700 fully reduced) in a given state.

**ECS analysis**

The ECS measurements were carried out using the Walz Dual-PAM 100 equipped with a P515/535 module. Each measurement was carried out in ambient air, using 4–5-week-old
plants grown under long-day conditions that had been dark-adapted for 30 min. It consisted of 5 min of red AL at 75 μmol photons m$^{-2}$ s$^{-1}$; a 1 s dark pulse at each different time point was used to record ECS$_t$. This represented the size of the light-induced pmf and was estimated from the total amplitude of the rapid decay of the ECS signal during the dark pulse, as described previously (Wang et al., 2015). The ECS$_t$ levels were normalized against a 515-nm absorbance change induced by a single turnover flash (ECS$_{ST}$), as measured in dark-adapted leaves before recording. This normalization allowed us to consider possible changes in leaf thickness and chloroplast density between leaves (Takizawa et al., 2008).

**In vivo photoreduction of thiol enzymes**

Photoreduction of Trx target enzymes in seedlings was determined using the free thiol-specific-modifying reagent, AMS (Thermo Fisher Scientific, USA) as described previously (Okegawa and Motohashi, 2015). Seedlings were dark-adapted for 8 h and exposed to light (80 μmol photons m$^{-2}$ s$^{-1}$) for up to 60 min. Samples were collected at the indicated time points and detected by western blot analysis. The reduction level of the proteins was quantified using Multi Gauge 3.1 software (Fujifilm, Japan) and presented as the ratio of reduced protein to total protein.

**Statistical analysis**

Calculations were performed on more than three independent biological replicates (see figure legends). Tukey multiple comparison test was used to determine significant differences among the materials tested (P<0.05).
Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: Trx f1 (At3g02730), Trx f2 (At5g16400), PGR1 (At4g03280), and PGR5 (At2g05620).

SUPPLEMENTAL DATA

Supplemental Figure S1. Visible phenotypes of the wild type (WT) and pgr1, pgr5, trx f1f2, pgr1 trx f1f2, and pgr5 trx f1f2 mutants.

Supplemental Figure S2. Light intensity dependence of PSI and PSII photosynthetic parameters in the wild type (WT) and pgr1, pgr5, trx f1f2, pgr1 trx f1f2, and pgr5 trx f1f2 mutants.

Supplemental Figure S3. Simultaneous analysis of PSI and PSII photosynthetic parameters during steady-state photosynthesis.

Supplemental Figure S4. Electrochromic shift (ECS) analysis in the wild type (WT) and pgr1, pgr5, trx f1f2, pgr1 trx f1f2, and pgr5 trx f1f2 mutants.

Supplemental Table S1. Primers used in this study.

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Conflict of interest

The authors have no conflict of interest to declare.

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Figure legends

Figure 1. Visible phenotypes of the wild type (WT) and pgr1, pgr5, trx f1f2, pgr1 trx f1f2, and pgr5 trx f1f2 mutants grown for 3 weeks under long-day conditions. A, Photographs of the plants. Bars indicate 10 mm. B, Fresh weights of seedlings. Each value is shown as the mean ± standard deviation (SD) of 10 independent replicates. Columns with the same letters are not significantly different between genotypes (Tukey-Kramer test, \( P < 0.05 \)). C, Chlorophyll content of seedlings, per unit fresh weight. Each value is the mean ± SD of three independent replicates. Columns with the same letters are not significantly different between genotypes (Tukey-Kramer test, \( P < 0.05 \)). D and E, Western blot analysis. Chloroplasts were fractionated into the stromal fractions (D) and thylakoid membranes (E). For the wild type, dilution series of proteins corresponding to 1.0 (100%), 0.5, 0.25, and 0.125 µg chlorophyll were loaded. Other mutants contained proteins corresponding to 1.0 µg chlorophyll in each lane.

Figure 2. Chlorophyll fluorescence analysis in the wild type (WT) and pgr1, pgr5, trx f1f2, pgr1 trx f1f2, and pgr5 trx f1f2 mutants. A, The maximum quantum yield of PSII \( (F_{v}/F_{m}) \). Each value is the mean ± SD of five independent replicates. Columns with the same letters are not significantly different between genotypes (Tukey-Kramer test, \( P < 0.05 \)). B, Photochemical quenching in the dark (qPd). qPd was determined after illumination at 50 µmol photons m\(^{-2}\) s\(^{-1}\) (growth light) for 15 min. Each value is the mean ± SD of five independent replicates. Columns with the same letters are not significantly different between genotypes (Tukey-Kramer test, \( P < 0.05 \)). C and D, Light-intensity dependence of the effective quantum yield of PSII \([Y(II)]\) and the non-photochemical quenching (NPQ) of chlorophyll fluorescence. Each value is the mean ± SD of five
independent replicates. E and F, Time courses of Y(II) and NPQ during the induction of
photosynthesis. The Y(II) and NPQ values were measured upon illumination at 75 µmol
photons m$^{-2}$ s$^{-1}$ for 20 min, followed by 8 min in the dark. Each data point represents the
mean ± SD ($n = 5$ independent plants). G, Linear electron transport in ruptured
chloroplasts. Y(II) was determined in ruptured chloroplasts at light intensities of 17 and
167 µmol photons m$^{-2}$ s$^{-1}$ (µE). Each value is the mean ± SD of three independent
chloroplast preparations. Columns with the same letters are not significantly different
between genotypes (Tukey-Kramer test, $P < 0.05$).

Figure 3. Simultaneous analysis of PSI and PSII photosynthetic parameters in the wild
type (WT) and $pgr1$, $pgr5$, $trx f1 f2$, $pgr1 trx f1 f2$, and $pgr5 trx f1 f2$ mutants.
Photosynthetic parameters were monitored under the induction of photosynthesis at a
light intensity of 75 µmol photons m$^{-2}$ s$^{-1}$ (µE). A, Photochemical quantum yield of PSI
[Y(I)]. B, Effective quantum yield of PSII [Y(II)]. C, Acceptor-side limitation of PSI
[Y(NA)]. D, Donor-side limitation of PSI [Y(ND)] E, Non-photochemical quenching
(NPQ) of chlorophyll fluorescence. Plants were dark-adapted for 30 min before
measurements. Each value is the mean ± SD of three independent replicates.

Figure 4. Electrochromic shift (ECS) analysis in the wild type (WT) and $pgr1$, $pgr5$, $trx$
$f1 f2$, $pgr1 trx f1 f2$, and $pgr5 trx f1 f2$ mutants. A, The total size of proton motive force
(pmf) determined as ECS$_t$/ECS$_{ST}$. ECS$_t$ is the light-dark difference in the ECS signal and
represents the magnitude of the pmf formed in the light. ECS$_{ST}$ signal was produced by a
single turnover light pulse using dark-adapted leaves. B, Proton conductivity of the
thylakoid membrane ($g_{H^+}$). Measurements were performed under the induction of
photosynthesis at a light intensity of 75 photons m\(^{-2}\) s\(^{-1}\). Each value is the mean ± SD of three independent replicates.

**Figure 5.** Photoreduction of thiol enzymes in the wild type (WT) and *pgr1, pgr5, trx f1f2, pgr1 trx f1f2*, and *pgr5 trx f1f2* mutants. Seedlings were illuminated at a light intensity of 80 µmol photons m\(^{-2}\) s\(^{-1}\) (µE) after a dark period of 8 h and collected at the indicated time points. The extracted proteins were modified with the thiol-reactive 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) and subjected to non-reducing SDS-PAGE. The redox states of ATP synthase CF\(_1\)-γ (A), FBPase (B), and SBPase (C) were detected by western blot analysis. The reduction levels of thiol enzymes were indicated as a percentage of the total protein that was reduced. Each value represents the mean ± SD of three independent replicates. Red, reduced; Ox, oxidized.
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Figure 1. Visible phenotypes of the wild type (WT) and pgr1, pgr5, trx f1f2, pgr1 trx f1f2, and pgr5 trx f1f2 mutants grown for 3 weeks under long-day conditions. A, Photographs of the plants. Bars indicate 10 mm. B, Fresh weights of seedlings. Each value is shown as the mean ± standard deviation (SD) of 10 independent replicates. Columns with the same letters are not significantly different between genotypes (Tukey-Kramer test, \( P < 0.05 \)). C, Chlorophyll content of seedlings, per unit fresh weight. Each value is the mean ± SD of three independent replicates. Columns with the same letters are not significantly different between genotypes (Tukey-Kramer test, \( P < 0.05 \)). D and E, Western blot analysis. Chloroplasts were fractionated into the stromal fractions (D) and thylakoid membranes (E). For the wild type, dilution series of proteins corresponding to 1.0 (100%), 0.5, 0.25, and 0.125 µg chlorophyll were loaded. Other mutants contained proteins corresponding to 1.0 µg chlorophyll in each lane.
Figure 2. Chlorophyll fluorescence analysis in the wild type (WT) and pgr1, pgr5, trx f1f2, pgr1 trx f1f2, and pgr5 trx f1f2 mutants. A, The maximum quantum yield of PSII (Fv/Fm). Each value is the mean ± SD of five independent replicates. Columns with the same letters are not significantly different between genotypes (Tukey-Kramer test, P < 0.05). B, Photochemical quenching in the dark (qPd). qPd was determined after illumination at 50 μmol photons m⁻² s⁻¹ (growth light) for 15 min. Each value is the mean ± SD of five independent replicates. Columns with the same letters are not significantly different between genotypes (Tukey-Kramer test, P < 0.05). C and D, Light-intensity dependence of the effective quantum yield of PSII [Y(II)] and the non-photochemical quenching (NPQ) of chlorophyll fluorescence. Each value is the mean ± SD of five independent replicates. E and F, Time courses of Y(II) and NPQ during the induction of photosynthesis. The Y(II) and NPQ values were measured upon illumination at 75 μmol photons m⁻² s⁻¹ for 20 min, followed by 8 min in the dark. Each data point represents the mean ± SD (n = 5 independent plants). G, Linear electron transport in ruptured chloroplasts. Y(II) was determined in ruptured chloroplasts at light intensities of 17 and 167 μmol photons m⁻² s⁻¹ (μE). Each value is the mean ± SD of three independent chloroplast preparations. Columns with the same letters are not significantly different between genotypes (Tukey-Kramer test, P < 0.05).
Figure 3. Simultaneous analysis of PSI and PSII photosynthetic parameters in the wild type (WT) and \( pgr1, pgr5, \) \( trx f1f2, pgr1 \) \( trx f1f2, \) and \( pgr5 \) \( trx f1f2 \) mutants. Photosynthetic parameters were monitored under the induction of photosynthesis at a light intensity of 75 µmol photons m\(^{-2}\) s\(^{-1}\) (µE). A, Photochemical quantum yield of PSI \([Y(I)]\). B, Effective quantum yield of PSII \([Y(II)]\). C, Acceptor-side limitation of PSI \([Y(NA)]\). D, Donor-side limitation of PSI \([Y(ND)]\). E, Non-photochemical quenching (NPQ) of chlorophyll fluorescence. Plants were dark-adapted for 30 min before measurements. Each value is the mean ± SD of three independent replicates.
Figure 4. Electrochromic shift (ECS) analysis in the wild type (WT) and pgr1, pgr5, trx f1 f2, pgr1 trx f1 f2, and pgr5 trx f1 f2 mutants. A, The total size of proton motive force (pmf) determined as ECS_t/ECS_ST. ECS_t is the light-dark difference in the ECS signal and represents the magnitude of the pmf formed in the light. ECS_ST signal was produced by a single turnover light pulse using dark-adapted leaves. B, Proton conductivity of the thylakoid membrane (g_{H^+}). Measurements were performed under the induction of photosynthesis at a light intensity of 75 photons m^{-2} s^{-1}. Each value is the mean ± SD of three independent replicates.
Figure 5. Photoreduction of thiol enzymes in the wild type (WT) and *pgr1*, *pgr5*, *trx f1f2*, *pgr1 trx f1f2*, and *pgr5 trx f1f2* mutants. Seedlings were illuminated at a light intensity of 80 µmol photons m$^{-2}$ s$^{-1}$ (µE) after a dark period of 8 h and collected at the indicated time points. The extracted proteins were modified with the thiol-reactive 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) and subjected to non-reducing SDS-PAGE. The redox states of ATP synthase CF$_1$-γ (A), FBPase (B), and SBPase (C) were detected by western blot analysis. The reduction levels of thiol enzymes were indicated as a percentage of the total protein that was reduced. Each value represents the mean ± SD of three independent replicates. Red, reduced; Ox, oxidized.
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