Transmission of reductive and oxidative cues from the photosynthetic electron transport chain to redox regulatory protein networks plays a crucial role in coordinating photosynthetic activities. The tight balance between these two signals dictates the cellular response to changing light conditions. While the role of reductive signals in activating chloroplast metabolism is well established, the role of their counterbalanced oxidative signals is still unclear, mainly due to monitoring difficulties. Here, we introduced chl-roGFP2-PrxΔCRR, a 2-Cys peroxiredoxin-based biosensor, into Arabidopsis thaliana chloroplasts to monitor the dynamic changes in photosynthetically derived oxidative signaling. We showed that chl-roGFP2-PrxΔCRR oxidation states reflected oxidation patterns similar to those of endogenous 2-Cys peroxiredoxin under varying light conditions. By employing a set of genetically encoded biosensors, we showed the induction of 2-Cys peroxiredoxin-dependent oxidative signals, throughout the day, under varying light intensities and their inverse relationship with NADPH levels, unraveling the combined activity of reducing and oxidizing signals. Furthermore, we demonstrated the induction of 2-Cys peroxiredoxin-derived oxidative signals during a dark–to–low-light transition and uncovered a faster increase in carbon assimilation rates during the photosynthesis induction phase in plants deficient in 2-Cys peroxiredoxins compared with wild type, suggesting the involvement of oxidative signals in attenuating photosynthesis. The presented data highlight the role of oxidative signals under nonstress conditions and suggest that oxidative signals measured by peroxiredoxin-based biosensors reflect the limitation to photosynthesis imposed by the redox regulatory system.

Significance

Identifying the intrinsic factors that regulate leaf photosynthetic rate may pave the way toward developing new strategies to enhance carbon assimilation. While the dependence of photosynthesis on the reductive activation of the Calvin–Benson cycle enzymes is well established, the role of oxidative signals in counterbalancing the reductive activity is just beginning to be explored. By developing 2-Cys peroxiredoxin-based genetically encoded biosensors, we demonstrated the induction of photosynthetically derived oxidative signals under habitual light conditions, a phenomenon typically masked by the dominance of the reductive power. Moreover, we unraveled the simultaneous activation of reductive and oxidative signals during photosynthesis induction phase and showed that 2-Cys peroxiredoxin activity attenuates carbon assimilation rates, demonstrating the restrictions imposed on photosynthetic performance by oxidative signals.

Sophisticated mechanisms enabling adjustments of light-capturing reactions and downstream metabolic processes have evolved in sessile plants, allowing them to reach optimal photosynthetic performance. Under low-light conditions, efficient photosynthetic activity and tight regulation of the reducing power distribution between essential metabolic pathways are required for optimal utilization of the available energy input. On the other hand, energy dissipation under high-light (HL) conditions is critical to avoid overproduction of harmful reactive oxygen species (1). Reductive and oxidative signals, both emanating from the photosynthetic electron transport chain (PETC), and their transfer to regulated thiol proteins play a significant role in linking the performance of photosynthesis to chloroplast transcription, translation, and metabolic processes, thereby enabling rapid acclimation to instantaneous changes in daytime photon fluxes (2–5).

Reductive signals in chloroplasts are mainly generated by the flux of electrons derived from ferredoxin to target proteins, via ferredoxin-dependent thioredoxin reductase (FTR), to thioredoxins (TRXs) (6) and via NADPH-dependent thioredoxin reductases C (NTRC) (7), thereby linking chloroplast metabolism to light availability and photosynthetic activity. Counteracting oxidative signals are mainly generated from electron flux through the water–water cycle (WWC). During the WWC, electrons are donated from photosystem I to molecular oxygen in the Mehler reaction, yielding superoxide radicals ($O_2^–$), which are then dismutated to molecular oxygen (O$_2$) and H$_2$O$_2$ in a reaction catalyzed by superoxide dismutase (8–10). The production of H$_2$O$_2$ through the WWC allows communication between the photosynthetic light reactions and downstream metabolic processes by transmitting oxidative signals to redox-regulated proteins (11–14).

The detoxification of photosynthetically produced H$_2$O$_2$ is mediated by chloroplast-targeted ascorbate peroxidases and 2-Cys peroxiredoxins (2-Cys Prxs) (15–18). The latter are highly abundant and remarkably efficient thiol peroxidases, displaying high catalytic efficiency of up to $10^8$ M$^{-1}$ s$^{-1}$ (19, 20). Electrons delivered from the PETC reduce 2-Cys Prx via the FTR/TRX pathways or through NADPH via NTRC activity (21–24). Therefore, the 2-Cys Prxs redox state is determined by the balance between photosynthetically produced reducing and oxidizing equivalents. Importantly, recent studies have demonstrated oxidative signal transmission, mediated by 2-Cys Prxs, from
H$_2$O$_2$ to target proteins at the onset of the light and dark periods and under low- and fluctuating-light conditions (25–30). Despite the considerable importance of the regulatory role of 2-Cys Prx, most of our current knowledge is based on measuring its oxidation state using Western blot analysis or examination of mutant lines performance, rendering it challenging to detect the transmission of oxidative signals in real time and with high temporal resolution.

Genetically encoded redox-sensitive green fluorescent protein (roGFp) probes enable, due to their high sensitivity and reversibility, systematic in vivo mapping of H$_2$O$_2$ production and the glutathione redox state ($E_{GSH}$), in different subcellular compartments (31–38). In vitro characterization of roGFp showed that its reduction is mediated by glutaredoxins, which catalyze the reversible electron flow between GSH and target proteins (34, 35). Recently, an H$_2$O$_2$ sensor based on a redox relay mechanism between Tsa2, a highly efficient Saccharomyces cerevisiae 2-Cys peroxiredoxin, and roGFp2 was developed (39). To increase the probe’s sensitivity to low H$_2$O$_2$ levels, a mutation in the resolving cysteine (C$_{60}$) of Tsa2 (roGFp2-Tsa2ΔC$_{60}$) was introduced, thus preventing competition between the Trx-mediated reduction of the Tsa2 moiety and roGFp oxidation. The resulting probe enabled the measurement of endogenous basal H$_2$O$_2$ levels in yeast and Chlamydomonas reinhardtii cells (39, 40); however, such a roGFp2 Prx-based probe has not yet been implemented to measure the dynamic oxidation of 2-Cys Prx in higher plants.

Here, we employed chl-roGFp2-PrxΔC$_{60}$, a 2-Cys Prx biosensor, to establish the role of 2-Cys Prx in light-dependent oxidative signal transmission in Arabidopsis plants. By parallel measurements of the endogenous 2-Cys Prx and chl-roGFp2-PrxΔC$_{60}$ oxidation states, we showed that 2-Cys Prx is directly regulated by light intensity. Furthermore, the dynamics of 2-Cys Prx-dependent oxidative signals, throughout the day, under various light conditions, were resolved by systematic monitoring of the probe’s redox state and showed a negative correlation with the availability of chloroplast NADPH. Finally, by using a set of genetically encoded biosensors, we unveil the combined effect of the reductive and oxidative pathways in fine-tuning photosynthetic activity.

**Results**

We sought to explore the direct effect of light intensity on the redox state of 2-Cys Prx. Upon reaction with peroxide, a Cys sulfenic acid intermediate is formed at the 2-Cys Prx peroxidatic Cys, which can further react with a resolving Cys of the second subunit, forming a head-to-tail 2-Cys Prx homodimer. Thus, the oxidation degree of 2-Cys Prx can be inferred by quantifying the transfer of plants from dark to normal growth light (GL, 120 μmol photons m$^{-2}$ s$^{-1}$; Fig. 1 A and B) resulted in a similar reduction of 2-Cys Prx catalytic sites upon dark-to-light transition for the two tested HL intensities, which stabilized at a higher oxidation state as compared with GL. A significant amount of fully oxidized 2-Cys Prx arose in plants exposed to 1,000 μmol photons m$^{-2}$ s$^{-1}$ (Fig. 1 D and E), suggesting that increased illumination enhanced 2-Cys Prx dimer formation, in which both catalytic sites participate in disulfide exchange reactions. Plant exposure to constant HL conditions or DCMU also led to higher levels of hyperoxidized 2-Cys Prx than GL, as measured using SO3 antibodies (SI Appendix, Fig. S3).

An apparent light-dependent oxidation response of 2-Cys Prx was observed under prolonged, physiologically relevant light conditions in which plants were exposed to gradually increasing light intensities, reaching a maximum value of 900 μmol photons m$^{-2}$ s$^{-1}$, followed by a gradual decrease in light intensities toward the dark period (Fig. 1 F and G and SI Appendix, Fig. S4). Interestingly, the major shift in oxidation was during the nonstressed light regime, in the transition from low (50 μmol photons m$^{-2}$ s$^{-1}$) to moderate (240–280 μmol photons m$^{-2}$ s$^{-1}$) light intensities, and vice versa, at the initiation and termination of the light phase (Fig. 1 F and G), implying its signaling role under habitual light conditions. Notably, accumulation of the Prx hyperoxidation form was not detected (Fig. 1 F, Lower), suggesting that a gradual increase in light conditions inhibits 2-Cys Prx overoxidation even while reaching HL conditions. These observations demonstrated that the 2-Cys Prx redox state is directly affected by the PETC and that its oxidation state under gradual changes in light intensities that mimic natural conditions in the field is firmly linked to changes in light intensities.

We recognized that the observed oxidation patterns of 2-Cys Prx in response to changing light intensities reflect the balance between its peroxidase activity and its reduction by TRXs. Therefore, to explicitly inspect the oxidative signals mediated by 2-Cys Prx activity, we generated, based on a previous work in yeast (39), a chl-roGFp2-PrxΔC$_{60}$ probe by genetically fusing roGFp2 to a mutated version of 2-Cys Prx A (BAS1), in which the resolving cysteine (C$_{60}$) was changed to alanine, impeding its TRX-dependent reduction (Fig. 2 A–C). BAS1 was fused at its N terminus to roGFp2, since initial attempts to fuse it at its C terminus resulted in probe cleavage (SI Appendix, Fig. S5). Interestingly, the cleaved roGFp obtained from the fusion of BAS1 at its C terminus was localized in the chloroplast (SI Appendix, Fig. S5), implying that the C terminus of BAS1 is prone to cleavage by specific chloroplast proteases.

A dynamic range of 5.6 was calculated between the fully oxidized (R$_{oxi}$ = 0.45) and fully reduced (R$_{red}$ = 0.08) forms of chl-roGFp2-PrxΔC$_{60}$, ensuring a sufficient signal-to-noise ratio (Fig. 2 D). Whole-plant ratiometric analysis showed a higher...
oxidation state of the chl-roGFP2-PrxΔC₉ probe compared with chl-roGFP2 under steady-state conditions (GL) (∼60% and ∼40%, respectively; Fig. 2 E–G), a phenomenon also observed with the roGFP2-Tsa2ΔC₉ probe (39, 40), indicating a direct effect of 2-Cys Prx on the roGFP moiety. Significant changes in the redox state of chl-roGFP2-PrxΔC₉ were visualized upon shifting plants from dark to different HL intensities (470, 700, or 1,000 μmol photons m⁻² s⁻¹), reaching its almost fully oxidized state within 5 min of transition to light (Fig. 2 E and G). In contrast, the unfused chl-roGFP2 probe was only moderately oxidized under HL conditions (Fig. 2 E and F). These findings demonstrate the enhanced light sensitivity of chl-roGFP2-PrxΔC₉ compared with chl-roGFP2, achieved by positioning BAS1 in close proximity to roGFP2. As both probes are subjected to reduction in a GSH/GRX-dependent manner, the comparison between their dynamic oxidation enables resolution of the effect on H₂O₂-dependent thiol oxidases mediated by BAS1.

We further investigated the daily dynamics of the chl-roGFP2-PrxΔC₉ oxidation state in comparison with chl-roGFP2 using an
automated system that allows for continuous measurements of fluorescence signals emanating from living plants expressing genetically encoded biosensors (Fig. 3 and Materials and Methods). Monitoring probe oxidation throughout the day further demonstrated the higher sensitivity of chl-roGFP2-PrxΔCR to light compared with chl-roGFP2. While both probes were oxidized upon transition from dark to light under all examined light conditions, significantly higher oxidation values were recorded under HL in chl-roGFP2-PrxΔCR (∼85 to 95%) as compared with chl-roGFP2 plants (∼65 to 70%; Fig. 3 B and C).

To characterize chl-roGFP2-PrxΔCR oxidation dynamics under a range of light intensities, we monitored its oxidation degree (OxD) in plants exposed to gradual changes in light intensities that mimic natural conditions, as described in Fig. 1 G. For comparison, the oxidation dynamics of the chl-roGFP2 and chl-roGFP2-Prx ΔCR probes were also monitored, as was NADPH availability, using the recently developed Arabidopsis NADPH sensor line (TKTP-iNAP4) (41, 42). As shown in Fig. 3 D, the four probes exhibited entirely different patterns. The chl-roGFP2-PrxΔCR

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**Fig. 2.** Light responsiveness of chl-roGFP2-PrxΔCR. (A) Schematic diagram of the gene cassette used to transform Arabidopsis plants. (B) Subcellular localization of chl-roGFP2-PrxΔCR in mesophyll cells, as detected by confocal microscopy. (C) Detection of the fused sensor protein by Western blot analysis using either anti-PRX or anti-GFP antibodies. (D) Fluorescence ratios (400/485) in plants expressing chl-roGFP2-PrxΔCR under steady-state conditions and following treatments with 100 mM DTT or 500 mM H2O2. Fluorescence emitted from 3-wk-old plants grown in soil was recorded using a plate reader. (E) Whole-plant ratiometric analysis of chl-roGFP2 and chl-roGFP2-PrxΔCR fluorescence under dark and 5 min following transfer from dark to different light intensities. Ratiometric analysis of plants treated with 100 mM DTT or 500 mM H2O2 is also presented. The roGFP2 fluorescence images were captured at 510 nm, following excitation at 400 nm and 465 nm. (F and G) Quantification of ratiometric images, presented as box plots.
OxD sharply increased from the light onset, reaching its full oxidation state (∼100%) after approximately 2 h, when the light intensity was ∼300 μmol photons m⁻² s⁻¹. A gradual decrease in OxD was only detected 14 h after light onset, when light intensities were dimmed again to ∼300 μmol photons m⁻² s⁻¹. We recorded a lower magnitude of oxidation of chl-roGFP2-PrxΔCR compared with chl-roGFP2-Prx, indicating the higher sensitivity of the latter to oxidative signals, achieved by the CR mutation. Notably, the oxidative patterns of chl-roGFP2-PrxΔCR and chl-roGFP2-Prx probes in response to changes in light intensities were similar to those of endogenous 2-Cys Prx (Figs. 1F and G and 3D), reinforcing the probe’s capabilities to detect 2-Cys Prx-mediated redox signals. We reasoned that the differences between chl-roGFP2-PrxΔCR and chl-roGFP2-Prx OxD reflected reducing equivalents transferred to 2-Cys Prx by the combined activity of NTRC and TRXs. In contrast to the oxidation patterns of chl-roGFP2-Prx and chl-roGFP2-PrxΔCR, the unfused chl-roGFP2 showed a gradual increase in OxD, reaching its highest level (65%) after 3 h, when the light intensity was ∼600 μmol photons m⁻² s⁻¹. From this point onward, a gradual...
decrease in chl-roGFP2 oxidation was observed, reaching its lowest OxD (42%) at the beginning of the dark period.

Strikingly, the redox state of chl-roGFP2-PrxΔCΔR negatively correlated with the dynamic changes in stromal NADPH availability, as recorded using the TKTP-iNAP4 lines. While the NADPH level was high and stable at night, a sharp decrease was detected under light, starting at light onset and reaching its lowest level at ~300 μmol photons m⁻² s⁻¹. A gradual increase in NADPH level was observed ~14 h after light onset, when light intensities were lowered, until it reached its highest level at the beginning of the night. These results imply that the availability of stromal NADPH governs the induction of photosynthetically derived oxidative signals.

The significant oxidation of chl-roGFP2-PrxΔCΔR under low to moderate light intensities (Fig. 3D), which matches the increasing recognition that 2-Cys Prx plays a regulatory role under low-light conditions (25–27, 43), drove us to further investigate the sensitivity of the chl-roGFP2-PrxΔCΔR redox state to low light intensity. To this end, plants were exposed to a gradual increase in light intensities, starting with ~22 μmol photons m⁻² s⁻¹ and increasing up to 330 μmol photons m⁻² s⁻¹ (Fig. 4A). An immediate reduction of chl-roGFP2 was observed at ~22 μmol photons m⁻² s⁻¹, followed by oxidation at a light intensity of ~44 μmol photons m⁻² s⁻¹, with oxidation reaching a maximum under 330 μmol photons m⁻² s⁻¹. In contrast, gradual oxidation of chl-roGFP2-PrxΔCΔR was observed with progression through the low-light gradient. Stromal NADPH availability negatively correlated with chl-roGFP2-PrxΔCΔR as in Fig. 3D. To further examine the induction of reductive and oxidative signals under low light, we monitored the probe’s oxidation pattern during prolonged exposure to low light. Interestingly, the chl-roGFP2-PrxΔCΔR and chl-roGFP2-Prx probes showed contrasting responses upon transition from dark to low light (Fig. 4B). While chl-roGFP2-PrxΔCΔR OxD slightly increased when plants were transferred to low light, chl-roGFP2-Prx underwent a significant decrease. This decrease continued for at least 2 h from light onset until stabilization at a lower OxD (35%). The differences between the OxD of roGFP2-PrxΔCΔR, chl-roGFP2-Prx, and the unfused chl-roGFP2 after the onset of low light demonstrate the high magnitude of reductive signals under low light intensities, emanating from TRXs and GSH/GRXs activity and which are attenuated by induced oxidative signals (Fig. 4C).

We hypothesized that the observed oxidative signals, upon transition from dark to low light, play a role in Calvin–Benson cycle (CBC) enzymes deactivation, thus substantially influencing carbon assimilation rates. To assess what extent 2-Cys Prxs limit photosynthesis, we measured the increase in carbon assimilation rates during the photosynthesis induction phase in dark-adapted mutants deficient in 2-Cys Prxs (2cpab) (28) and wild-type (WT) plants. Notably, while upon transition to low light, a considerable lag in the attainment of photosynthesis steady-state was observed in WT plants, a significantly faster induction of photosynthesis was detected in 2cpab plants (Fig. 4D and SI Appendix, Fig. 5B), pointing to the inhibitory effect of 2-Cys Prxs activity during the photosynthesis induction phase. Furthermore, this fast induction was not detected in plant lines mutated in prxQ and prxIIE, two additional chloroplast-targeted Prxs (44, 45) (Fig. 4D), suggesting the exclusive role of 2-Cys Prxs in determining the limitation to photosynthesis during its induction phase. The limitation to photosynthesis mediated by 2-Cys Prx was also demonstrated in plants mutated in NTRC, an essential component of the reductive signal pathway (Fig. 4E). As shown in Fig. 4E, ntrc plants showed a significantly lower increase in carbon assimilation rate than WT upon shifting of plants from dark to low light, emphasizing the dependency of the photosynthesis induction phase on the redox regulatory network. Notably, this effect was partially recovered in ntrc-Δ2cp triple mutant plants, which express severely low levels of 2-Cys Prxs (Fig. 4E). These results are in agreement with the reported suppression of ntrc growth phenotype by decreased levels of 2-Cys Prxs (46).

The fast induction of photosynthesis in 2cpab compared with WT plants was also observed upon returning of moderate-light-adapted plants from short exposure of low light (5 min) back to moderate light intensities (Fig. 4F). In contrast, when 2cpab and WT plants were exposed to a brief low light period of 1 min (SI Appendix, Fig. S9) no differences were observed, indicating a time-dependent buildup of the 2-Cys Prx inhibitory effect. These results demonstrate the role of the redox regulatory system in shaping carbon assimilation rates under dynamic light conditions and the limitation to photosynthesis imposed by 2-Cys Prxs activity.

**Discussion**

It had been established that photosynthesis is dependent on incoming light not only as an energy source to drive electron transfer but also for reducing regulatory disulfides in stromal proteins, allowing the adjustment between chloroplast metabolism and photosynthetic activity (6). Besides generating reductive fluxes, photosynthetic light reactions produce oxidizing agents such as hydrogen peroxide, chiefly through the WWC (ref. 9 and Fig. 5). Accordingly, the in vivo redox state of regulatory thiols is dependent on the sum of reductive and oxidizing signals derived from the photosynthetic reducing and oxidizing activity, respectively (Fig. 5). While classical redox biology approaches allow the capturing of the redox state of a given protein/thiol, discerning the biological context of the reductive and oxidative pathways is still challenging. On the basis of the increasing recognition that chloroplast-targeted 2-Cys Prxs is capable of transferring oxidative signals to target proteins (25–29, 46) and based on the recently developed roGFP2-Tsa2ΔC probe in yeast (39), we developed chl-roGFP2-PrxΔCΔR in order to define the exact light regime and time frame in which oxidizing signals are transmitted.

Interestingly, by using a set of genetically encoded biosensors (Fig. 5), we demonstrated that oxidizing activity governed by 2-Cys Prxs is prevalent under habitual growth conditions. This postulation is based on the differences in oxidation patterns observed for the chl-roGFP2-PrxΔCΔR and two additional probes monitored in this study, chl-roGFP2-Prx and chl-roGFP2 (Figs. 3D and 4A–C). Since the actual measured redox state of each probe is affected similarly to natural redox-regulated proteins, by the combining activity of reductive and oxidative signals, the comparison between the oxidative states of these probes allows deducing 2-Cys Prxs oxidizing activity. This concept is clearly demonstrated in the response of the three biosensors to low light conditions (Fig. 4 A–C). The immediate reduction observed for the chl-roGFP2 and roGFP2-Prx probes signifies the dominant reductive activity during the dark-to-low-light transition. Most strikingly, the relatively stable oxidation state of chl-roGFP2-PrxΔCΔR at the onset of low light compared with the reduction observed in the chl-roGFP2 and chl-roGFP2-Prx probes, at the same conditions, suggest the induction of oxidative signals under low light intensities, attenuating the reductive signals emanated from GSH/GRXs, FTR/TRx, and NTRC activities. These results are in agreement with the observations of
transmitted oxidative signals through ACHT1/4 under low to moderate light intensity (25, 26).

Tracking the in vivo dynamics of oxidative thiol modulations is central to understanding the role of redox regulation in adjusting metabolic activity (47). Exploring the diurnal redox dynamics of the CBC enzymes, FBPase and SBPase, showed a change from fully oxidized to an almost fully reduced state of FBPase during gradual increases in light intensity from dark to 350 μmol photons m⁻² s⁻¹ (48). Intriguingly, the observed light range in which FBPase reduction occurred matched the range of light-induced changes in 2-Cys redox signaling, as observed either by direct measurements of the 2-Cys Prx redox state or by using the chl-roGFP2-PrxΔCᵣ (Figs. 1 F and G, 3D, and 4A). Considering the sensitivity of FBPase to 2-Cys Prx-mediated oxidation (29, 30), these results suggest that the reductive and oxidative pathways are interconnected in regulating the activation state of carbon assimilation enzymes under low- and moderate-light conditions (Fig. 5). This integrative

### Fig. 4. chl-roGFP2-PrxΔCᵣ reports on the induction of oxidative signals under low light. (A) Daily dynamic changes in the OxD of chl-roGFP2-PrxΔCᵣ and chl-roGFP2, and in TKTP-NAP4 ratios, in plants exposed to a gradual increase in light intensities (low to moderate), followed by an equivalent decrease, are presented. The applied light intensities are depicted as a dashed line. Monitoring was performed in 3-wk-old plants grown in 12-well plates. For each line, between 24 and 40 plants divided into three to five independent plates were analyzed and consolidated in a “sliding window” (n = 3 to 5) display. Values represent means ± SE. The color bar denotes the light conditions: black, dark; yellow, light. (B) Dynamic changes in chl-roGFP2-PrxΔCᵣ, chl-roGFP2-Prx, and chl-roGFP2 OxD at the onset of low light (22 μmol photons m⁻² s⁻¹). (C) A proposed model of the oxidation and reduction powers that shape the oxidation state of the chl-roGFP2-PrxΔCᵣ, chl-roGFP2-Prx, and chl-roGFP2 probes. Figure created with BioRender (https://biorender.com/). (D and E) Carbon assimilation rate was measured in dark-adapted WT and mutants plants (2cpab, prxQ, prxEII, ntrc, and ntrc-Δ2CP) upon exposure to low light (22 μmol photons m⁻² s⁻¹). As differences in dark respiration rates were observed between the mutant lines, gas exchange data were normalized to the dark values. Values for the carbon assimilation are presented as means of four or five pots with four plants in each ± SE. The color bar denotes the light conditions: black, dark; yellow, light. (F) Carbon assimilation rate was measured in moderate light (330 μmol photons m⁻² s⁻¹) adapted WT and 2cpab plants upon exposure to 5 min of low light (22 μmol photons m⁻² s⁻¹) followed by moderate light (330 μmol photons m⁻² s⁻¹). Gas exchange data were normalized to values obtained during low light at the beginning of the experiment. Values for the carbon assimilation are presented as means of four pots with four plants in each ± SE. The color bar denotes the light conditions: yellow, low light; orange, moderate light.
view suggests that eliminating oxidative signals will result in a higher reduction state of CBC enzymes and consequently higher carbon assimilation rates. Indeed, the high reduction state of FBPase recently reported in \textit{2cpab} plants and the rapid increase in carbon assimilation rates observed exclusively in \textit{2cpab} and not in \textit{prxQ} or \textit{prxQIE} mutant plants (Fig. 4D) corroborates this view and points to the role of 2-Cys Prx in attenuating carbon assimilation rates. Furthermore, the inverse relationship between NADPH level and the induction of oxidative signals (Figs. 3D and 4A) suggests a feedback mechanism in which NADPH consumption may trigger oxidative signals, leading to inhibition of CBC enzyme activities and a consequent decrease in carbon assimilation rates.

In contrast to the rapid increase in carbon assimilation rates observed in \textit{2cpab} plants during the photosynthesis induction phase, lower steady-state carbon assimilation rates under HL in \textit{2cpab} plants compared with WT were recently reported (49) and also observed in this study (SI Appendix, Fig. S10). Thus, 2-Cys Prx affects photosynthesis performance by two means. Under nonstress conditions, 2-Cys Prx redox signaling, which is involved in attenuating carbon assimilation rates, is prevalent. On the contrary, under steady-state HL conditions, in which a higher rate of peroxide production is expected, the antioxidant activity rather than the signaling role of 2-Cys Prx is dominant and protects the photosynthetic apparatus from oxidative damage (16). The contrasting effect of 2-Cys Prx activity on photosynthesis under HL and nonstress conditions raises the possibility that its inhibitory effect on photosynthesis was retained, through plant evolution and breeding, due to its protecting role under HL.

Due to the heterogeneous organization and spatial arrangement of crop canopies, leaves lower in the canopy experience sporadic light conditions and breeding, due to its protecting role under HL. Moreover, the inverse contrast effect of 2-Cys Prx activity on photosynthesis under HL and nonstress conditions raises the possibility that its inhibitory effect on photosynthesis was retained, through plant evolution and breeding, due to its protecting role under HL.

Carbon assimilation rates (50). The inherent redox changes that stimulate the activation and deactivation of CBC enzymes link carbon fixation to electron transport activity and energy flux but also govern limitation to optimal canopy assimilation under fluctuating light intensities. Given the role of 2-Cys Prx in slowing photosynthesis rates during its induction phase (Fig. 4) and the impact of the induction phase on the net cumulative carbon assimilation (51), our data suggest that the chl-roGFP2-PnxΔCR probe estimates the degree of limitation of photosynthesis imposed by oxidative signals in plants, laying the foundation for increasing photosynthesis efficiency through redox modulations.

Materials and Methods

Plant Material, Growth Conditions, and Treatment. \textit{Arabidopsis thaliana} WT (ecotype Columbia, Col-0), \textit{prxQ} (Salk_070860C), and \textit{prxQIE} (Salk_203706C) were obtained from ABRC. The mutants \textit{2cpab}, \textit{nrc}, and \textit{nrc ΔTCP} were obtained from Francisco Javier Cejudo, Universidad de Sevilla, Sevilla, Spain (28, 46). WT and mutants plants were grown in a greenhouse under a 16/8 h light/dark cycle. Immunobass, redox imaging, and thiol labeling experiments were performed on whole, 3-wk-old \textit{Arabidopsis} plants. For the DCMU treatment, 3-wk-old plants were sprayed with 150 μM DCMU (D2425-100G, Sigma) in the dark phase, 1 h before the onset of the light period.

Construction of chl-roGFP2-Pnx and chl-roGFP2-PnxΔCR Probes. Fusion probes (chl-roGFP2-Pnx, chl-roGFP2-PnxΔCR, and chl-PnxΔCR-roGFP2) were synthesized with codons optimized for \textit{Arabidopsis} expression (Geneviz). Chloroplast targeting was achieved by using the 2-Cys peroxidexin A signal peptide. The genetically fused sequences were cloned into the plant cloning vector \textit{pART7}, using the XhoI and HindIII restriction enzymes. The whole construct, including the GmMV 35S promoter and nptII terminator, was then cloned into the binary vector \textit{pART27}, using the NotI restriction enzyme. The \textit{pART27} plasmids containing the chl-roGFP2-Pnx, chl-roGFP2-PnxΔCR, and chl-PnxΔCR-roGFP2 probe constructs were transformed into GV3101 \textit{Agrobacterium tumefaciens}. Transformation of \textit{Arabidopsis} thaliana (Col-0) was then performed by floral dip (52). Transformant lines were selected based on kanamycin resistance and the chl-roGFP2 fluorescence signal.

Screening of chl-roGFP2-Pnx and chl-roGFP2-PnxΔCR DNA Insertion in Transformed Plants. The substitution of the resolving cysteine (C) by alanine in chl-roGFP2-PnxΔCR plants was verified by DNA insertion screening. DNA extraction and PCR amplification were carried out using REDExtract-N-AmpTM Plant PCR kit (Sigma-Aldrich). The sequence of both primers, forward and reverse, were as follows: forward primer: CATCCAAGAAGACCGATGAGATGCTGCTTACAGAAAATATTCTTCTGACAC; reverse primer: TTGGAGAGGAAATAGTTCTTGGCAGCAC. The forward primer was designed to form HindIII restriction site sequence in the PCR products, thus allowing to distinguish between \textit{Pnx} and \textit{PnxΔCR}. The PCR products were digested with HindIII and run in an 80-bp product for \textit{PnxΔCR} (SI Appendix, Fig. S6): PCR product for Pnx (80 bp): CATCCAAGAAGACCGATGAGATGCTGCTTACAGAAAATATTCTTCTGACAC; PCR product for PnxΔCR (101 bp): CATCCAAGAAGACCGATGAGATGCTGCTTACAGAAAATATTCTTCTGACAC. The PCR product for PnxΔCR was digested with HindIII and run in an 80-bp product for \textit{PnxΔCR} (SI Appendix, Fig. S10): PCR product for PnxΔCR (80 bp): CATCCAAGAAGACCGATGAGATGCTGCTTACAGAAAATATTCTTCTGACAC.

\textit{N-Ethylmaleimide} (NEM)-Based Redox Labeling. Protein extracts were prepared, using a hand homogenizer, in 1 mL cold 10% trichloroacetic acid (TCA) (dissolved in water) to retain the in vivo thiol oxidation status of proteins. Proteins were precipitated for 30 min on ice, in the dark, and centrifuged at 19,000 \texttimes g for 20 min at 4 °C. The pellet was then washed four times with 100% cold acetone. After removal of the residual acetone, the pellet was resuspended in urea buffer [8 M urea, 100 mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.2, 1 mM ethylenediaminetetraacetic acid (EDTA), 2% (wt/vol) sodium dodecyl sulfate (SDS), and protease inhibitors mixture (Calbiochem)] which contains 100 mM NEM (E3876; Sigma) dissolved in ethanol. Samples were then incubated for 30 min, at room temperature, followed by centrifugation (19,000 \texttimes g, 20 min, 4 °C) and washed (four times) with 100% cold acetone. The dry pellets were resuspended in the urea buffer without NEM. The sample buffer (x3) contained 150 mM Tris-HCl, pH 6.8, 6% (wt/vol) SDS, 30%
glycerol, and 0.3% pyronin Y. Gel fractionation was carried out without a reducing agent, on a precast 4 to 15% polyacrylamide gel. Fractionated proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad), using the Trans-Blot Turbo Transfer System (Bio-Rad) with Trans-Blot Turbo Midi Transfer Packs. The membrane was incubated with anti-PRX antibody (1:1,000) (kindly provided by Avihai Danon, Weizmann Institute of Science, Rehovot, Israel) or an anti-peroxideroxidin-303 antibody (1:2,000, ab16830; Abcam), both followed by anti-rabbit horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) (1:20,000; Agrisera). Chemiluminescence was detected using the Advanced Molecular Imager HF (Spectral Ami-HT; Spectral Instruments Imaging, LLC).

mPEG-Based Redox Labeling. Protein extract was prepared, using a hand homogenizer, in 1 mL cold 10% TCA (dissolved in water). Proteins were precipitated for 30 min, on ice, in the dark, and then centrifuged at 19,000 × g for 20 min at 4 °C. The pellet was washed four times with 100% cold acetone, after which residual acetone was removed and the pellet was resuspended in the urea buffer with NEM. The samples were incubated for 30 min at room temperature. Samples were then reduced by addition of 100 mM dithiothreitol (DTT) (60 min, room temperature). TCA (10%) was added and samples were precipitated for 30 min, on ice, in the dark. The TCA-treated extracts were centrifuged at 19,000 × g, 4 °C for 20 min, and supernatants were removed and the pellets were washed three times with 100% acetone. The dry pellets were resuspended in urea buffer containing 10 mM mPEG (molecular weight 5,000 g/mol; 63187; Sigma), and incubated at 28 °C for 2 h. The reaction was stopped by adding an equal volume of sample buffer containing 50 mM DTT. Samples were separated by 10% SDS polyacrylamide gel electrophoresis (PAGE) and then transferred to a polyvinylidene fluoride membrane (Bio-Rad) under the same conditions described above. The membrane was incubated with anti-PRX antibody (1:1,000) followed by anti-rabbit HRP-conjugated IgG (1:20,000).

Probe Detection Using Western Analysis. Experiments were performed on 10-d-old seedlings. Whole plant contents were extracted with extraction buffer (20 mM Tris, pH 8.0, 1 mM EDTA, and 50 mM NaCl) and then centrifuged at 19,000 × g, for 20 min, at 4 °C. Gel fractionation of 100 μg extracted protein was carried out by reducing 10% SDS-PAGE. Fractionated proteins were then transferred onto a polyvinylidene fluoride membrane, which was then incubated with anti-PRX antibody (1:1,000) or anti-GFP (Abcam) antibody (1:5,000), and then with anti-rabbit HRP-conjugated IgG (1:20,000).

Confocal Microscopy. Confocal microscopy analysis was carried out on 2-wk-old transgenic chl-roGFP2–, chl-roGFP2-PnxΔCr, and chl-PnxΔCr-roGFP2-expressing plants. Images were captured with a Leica TCS SP8 confocal system (Leica Microsystems) and the LAS X Life Science Software, while using a HC PL APO ×40/0.10 objective. To capture chl-roGFP2 fluorescence, samples were excited at 488 nm and emission was measured at 500 to 520 nm. For chlorophyll fluorescence, excitation was at 488 nm and emission was at 670 nm. Merged images were generated using Fiji software.

roGFP2 Fluorescence Measurements and Analysis. Redox imaging was performed on 3-wk-old transgenic chl-roGFP2– and chl-roGFP2-PnxΔCr-expressing plants according to ref. 53. Whole-plant roGFP2 fluorescence was detected using an Advanced Molecular Imager HT, and images were taken using the AMIview software. For roGFP2 fluorescence images, excitation was at 465 nm and 405 nm, followed by emission at 510 nm. Chlorophyll fluorescence, which was used to select plant tissue pixels, was measured following excitation at 405 nm and emission at 670 nm. For each probe, images were taken under the same settings of light-emitting diode intensities and exposure time. Ratiometric images were created by dividing, pixel by pixel, the 405-nm image by the 465-nm image and displaying in false colors. Images were processed using a custom-written MATLAB script. For calibration of the probe response, detached plants were immersed in 0.5 M H2O2 or 100 mM DTT, and ratiometric images for fully oxidized and fully reduced states, respectively, were obtained.

Daily roGFP2 fluorescence was measured using a Tecan Spark multimode microplate reader (54). For all probes (chl-roGFP2, chl-roGFP2-Pnx, and chl-roGFP2-PnxΔCr), roGFP fluorescence was measured using excitation at 480 nm/20 and 400 nm/20, followed by emission at 520 nm/10; gain values were adjusted to avoid signal saturation. For chlorophyll detection, 400 nm/20 was applied for excitation, followed by 670 nm/40 for emission. For automatic detection of chl-roGFP2 signals, 10-d-old plants expressing the roGFP2-based probes or WT plants were transferred to solid petri plates in 12-well plates. The plates were placed in a growth chamber (Fytoptoscope FS-S1-4600; Photon Systems Instruments) under normal growth conditions for another 5 d before the start of the experiment. An automated robot (KINEDeX KX-01467; PAA Automation) controlled by a self-compiled program (Overlord; PAA Automation) inserted the plates into the plate reader to measure chl-roGFP2 fluorescence. For the plate-reader analysis, a nine-by-nine-pixel matrix was formed for each well. Chlorophyll fluorescence was detected to create a chlorophyll mask, which was then used to choose pixels that returned a positive chlorophyll fluorescence signal, and only those pixels were considered for the roGFP analysis. The average signals of nonfluorescent plants (WT) was calculated, and the values were deducted from the values detected in the chl-roGFP2 fluorescence analysis. A similar experimental setup was used to measure the fluorescence emitted from the TKTP-iNAP4 and TKTP-iNAPc-expressing lines. The pH-corrected ratio for TKTP-iNAP4 was calculated by dividing the fluorescence values obtained from the TKTP-iNAP4 line by those obtained from the TKTP-iNAPc line, according to ref. 42. To determine sensor OxD, detached roGFP-based probe-expressing and WT plants were immersed in 0.5 M H2O2 or 100 mM DTT and fluorescence was measured. roGFP2 OxD was calculated based on the fluorescence signal according to the following equation (34):

\[
OxD_{roGFP} = \frac{R - R_{red}}{\left(\frac{485\text{nm}}{485\text{nm}}\right)(R_{ox} - R) + \left(\frac{R - R_{red}}{485\text{nm}}\right)}
\]  

where \( R \) represents the ratio (400/485) at each point in the experiment, \( R_{ox} \) represents the ratio under fully oxidized conditions, \( R_{red} \) represents the ratio under fully oxidized conditions, \( f_{485\text{nm}} \) represents the fluorescence emitted at 520 nm when excited at 485 nm under fully oxidized conditions, and \( f_{485\text{nm}} \) represents the fluorescence emitted at 520 nm when excited at 485 nm under fully reduced conditions. A self-written MATLAB script was used to analyze the data collected by the plate reader. In addition, experimental metadata such as plant lines, light irradiance, temperature, relative humidity, and CO2 concentration were also collected and added to the output file.

Gas Exchange Measurements. Gas exchange measurements were performed using the LI-6800 (LI-COR). Four plants were planted in LI-COR 65 mm pots (610-09646) and analysis was done using the LI-6800 small plant chamber (6800-17). Total leaf area was calculated via image analysis using the Ami HT Imager and MATLAB.

Statistics. The data analyses were performed using the JMP statistical software. Box plots were created using the BoxPlotR tool (55).

Data Availability. All study data are included in the article and/or SI Appendix. ACKNOWLEDGMENTS. We would like to thank the late Avihai Danon for his critical comments on the manuscript and for kindly providing us with the anti-Cys Prx antibody. We thank Francisco Javier Cejudo for kindly providing 2Cpab ntrC and ntrCΔ2CP mutants. We thank Wallace Boon Leong Lim, who kindly provided the TKTP-iNAP4 and TKTP-iNAPc Arabidopsis lines. We thank Bruce Morgan for his kind help in initiating this project. We thank Gyu Golan for developing the PCR-based screen used to distinguish between the chl-roGFP2-Pnx and chl-roGFP2-PnxΔCr lines. This research was supported by Israel Science Foundation Grants 82617, 82717, and 177921.

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