Metabolic and diffusional limitations of photosynthesis in fluctuating irradiance in Arabidopsis thaliana

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A better understanding of the metabolic and diffusional limitations of photosynthesis in fluctuating irradiance can help identify targets for improving crop yields. We used different genotypes of Arabidopsis thaliana to characterise the importance of Rubisco activase (Rca), stomatal conductance (gs), non-photochemical quenching of chlorophyll fluorescence (NPQ) and sucrose phosphate synthase (SPS) on photosynthesis in fluctuating irradiance. Leaf gas exchange and chlorophyll fluorescence were measured in leaves exposed to stepwise increases and decreases in irradiance. rwt43, which has a constitutively active Rubisco enzyme in different irradiance intensities (except in darkness), showed faster increases than the wildtype, Colombia-0, in photosynthesis rates after step increases in irradiance. rca-2, having decreased Rca concentration, showed lower rates of increase. In aba2-1, high gs increased the rate of change after stepwise irradiance increases, while in C24, low gs tended to decrease it. Differences in rates of change between Colombia-0 and plants with low levels of NPQ (npq1-2, npq4-1) or SPS (spsa1) were negligible. In Colombia-0, the regulation of Rubisco activation and of gs were therefore limiting for photosynthesis in fluctuating irradiance, while levels of NPQ or SPS were not. This suggests Rca and gs as targets for improvement of photosynthesis of plants in fluctuating irradiance.

In physiological research, plants are often studied under constant environmental conditions. However, plants grow in a variable environment, with changes occurring in the time range of seconds or less1. Of the factors important for net photosynthesis (A\textsubscript{n}), irradiance changes most quickly2, causing a lag between changes in irradiance and changes in A\textsubscript{n} due to the slower regulation of photosynthesis. The lag decreases light-use efficiency relative to the steady state and transiently increases excess irradiance, possibly harming the photosynthetic apparatus. Leaves engage various mechanisms in response to fluctuating irradiance. Among the best known mechanisms are the regulation of enzymes of carbon fixation and sucrose metabolism, non-photochemical energy dissipation and stomatal conductance (gs). Although difficult to measure, cyclic electron transport may be another important mechanism (recently reviewed by Yamori and Shikanai4), due to a potential regulatory role and the balance of ATP versus NADPH production. During induction of photosynthesis in leaves adapted to darkness or low irradiance, the slow regeneration of ribulose-1,5-bisphosphate (RuBP) is typically most limiting until 60 seconds after illumination. Thereafter, both the slow carboxylation due to partially inactive Rubisco (time to full activation: ~10 minutes) and slow stomatal opening (10–60 minutes) can limit the rate at which photosynthesis increases. Thus, the slow rate of change of these mechanisms results in the lag between changes in irradiance and A\textsubscript{n} and the resulting reduction of plant productivity. Reductions in assimilation due to these physiological limitations can be up to 35% per day (subject to light environment and genotype), and understanding them better may pave the road towards higher yields.1,2

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Our understanding of the metabolic constraints of photosynthesis in fluctuating irradiance (hereafter: ‘dynamic photosynthesis’) have mainly come from biochemical studies, with less use being made of genetic diversity. Naturally occurring ecotypes, mutations, cultivars and genetically modified accessions offer a range of genotypes with specific properties, that could be used to study dynamic photosynthesis. *Arabidopsis thaliana* possesses a wide, well documented genotypic diversity, which has been extended by selecting for mutations and by transgenic modifications.

Rubisco catalyses CO$_2$ assimilation and its activation limits $A_o$ after irradiance increases. In the chloroplast stroma, several inhibitory compounds are present and bind to Rubisco. To maintain sufficient Rubisco activity, these inhibitors must be removed from the active sites by the ATPase Rubisco activase (Rca). In *Arabidopsis thaliana*, there are two isoforms of Rca, the larger $\alpha$-isoform and the smaller $\beta$-isoform. In plants containing both isoforms, redox-regulation of the $\alpha$-isoform affects the ADP sensitivity of the holoenzyme (composed of both isoforms). In low irradiance (i.e. high ADP/ATP ratio), the $\alpha$-isoform is less active and the rate of overall Rubisco activation is low. Since Rca is a central regulator of Rubisco activity, how these isoforms, or their concentration affect dynamic photosynthesis is an important yet unresolved question.

After CO$_2$ assimilation by Rubisco, a fraction of the triose phosphates leaves the chloroplast in exchange for orthophosphate (P$_i$) from the cytosol. In the cytosol, triose phosphate is converted to sucrose, and sucrose phosphate synthase (SPS) plays a central role in this pathway. In certain circumstances, such as photosynthetic induction in saturating CO$_2$, irradiance-dependent activation of SPS can be slower than that of Calvin cycle enzymes, making the Calvin cycle transiently P$_i$-limited. Furthermore, after irradiance decreases, an overshoot in sucrose synthesis can transiently drain metabolites from the Calvin cycle, transiently decreasing $A_o$. Plants with reduced SPS concentration may therefore exhibit slower increases in $A_o$ after irradiance increases, and a smaller transient dip in $A_o$ after irradiance decreases.

Leaves protect themselves from absorbed irradiance that is in excess of the capacity of photochemistry using non-photochemical quenching (NPQ). This protection, however, may come at a price. Sustained high levels of NPQ after irradiance decreases may result in transient limitations of the quantum efficiency of photosystem II for electron transport ($\phi$$_{psII}$). Model calculations indicate that slow relaxation of NPQ could decrease canopy photosynthesis by -13–24%. NPQ has been shown to limit $A_o$ in genotypes with faster NPQ buildup after irradiance increases or slower NPQ relaxation after irradiance decreases. Thus, genotypes with constitutively low NPQ may have increased dynamic photosynthesis rates, principally as a result of less limitation on $A_o$ following a decrease in irradiance.

In many plants, stomata open when irradiance increases. Typically, stomatal opening is slow, transiently limiting $A_o$ during the irradiance increase. Genotypes with constitutively high $g_o$ may not experience this limitation, and may therefore be more productive in environments with a high proportion of fluctuating irradiance, provided that water is not limiting.

We used several genotypes, i.e. plants containing point mutations, transformants, T-DNA insertion lines (SALK lines) and naturally occurring accessions of *A. thaliana*, to analyse how metabolic (Rubisco activation, sucrose synthesis, NPQ) and diffusional ($g_o$) limitations affect dynamic photosynthesis. In addition to measuring their steady-state photosynthetic irradiance and CO$_2$ responses, we exposed these genotypes to stepwise increases and decreases in irradiance, while measuring gas exchange and chlorophyll fluorescence. To investigate the effects of Rca regulatory properties or concentrations, we used the transformant *rwt43* (lacks the $\alpha$-isoform of Rca and is therefore ADP-insensitive) and the mutant *rca-2*, which is due to a leaky allele mutation (decreased Rca concentration). To analyze the effect of SPS, we studied the T-DNA mutant line *spat1* (80% reduction in maximum SPS activity). The effect of low NPQ was investigated by using *npq4-1* (lacks PsbS, greatly diminishing NPQ) and *npq1-2* (lacks zeaxanthin deepoxidase and therefore violaxanthin, diminishing NPQ). Effects of high and low $g_o$ were analyzed by using *aba2-1* (impaired abscisic acid (ABA) synthesis, leading to constitutively high $g_o$) and the natural accession C24 (low $g_o$), respectively. The accession Col-0 is the wildtype background to all mutants and transformants used in this study and acts as a control line. This study indicates that wildtype isofrom composition and amount of Rca, as well as $g_o$ limit dynamic photosynthesis in *A. thaliana*, while wildtype levels of SPS and NPQ do not.

**Results**

Steady-state responses to irradiance and CO$_2$ confirm genotypic effects on Rubisco activation state, sugar metabolism and stomatal conductance. To characterize the steady-state behaviour of the different *A. thaliana* genotypes we measured their responses to irradiance and leaf internal CO$_2$ concentration ($C_i$). Rates of $A_o$ in Col-0 were comparable to studies using plants grown under similar conditions. In the mutant containing less Rca, *rca-2*, irradiance-saturated $A_o$ was lower than for Col-0, and saturation occurred around 600 $\mu$mol m$^{-2}$ s$^{-1}$ (Fig. 1a). The lower $C_i$ response on $A_o$ in *rca-2* (Fig. 1b) resulted in significantly decreased maximum carboxylation rate by Rubisco ($V_{cmax}$; -23%), maximum rate of electron transport ($J_{max}$; -14%) and maximum rate of triose phosphate utilisation (TPU; -7%) compared to Col-0 (Table 1). Assimilation rates in the mutant lacking the $\alpha$-isoform of Rca, *rwt43*, exhibited similar irradiance and $C_i$ responses as in Col-0 (Fig. 1). In the mutant with less SPS (*spat1*), $A_o$ did not differ from Col-0 in its irradiance response (Fig. 1a), but was strongly reduced at high $C_i$ (Fig. 1b), resulting in decreased $J_{max}$ (-14%) and TPU (-23%). The ABA-deficient mutant, *aba2-1*, showed larger irradiance- and CO$_2$-saturated photosynthesis rates compared to Col-0, while the accession C24 showed the opposite (Fig. 1c,d). Some parameters derived from $C_i$ response curves were therefore larger in *aba2-1* ($J_{max}$: +18%; TPU: +19%), while they were smaller in C24 ($V_{cmax}$: -17%; $J_{max}$: -20%; TPU: -22%). The supply lines emphasize differences in $g_o$ between C24, Col-0 and *aba2-1*: the steeper the slope, the smaller the difference between external CO$_2$ concentration ($C_o$) and $C_i$ and the larger $g_o$. Irradiance and $C_i$ responses of photosynthesis of low-NPQ mutants (*npq1-2, npq4-1*) were similar to Col-0 (Fig. 1e,f), except for lower $J_{max}$ in *npq4-1* (-7%). The response of quantum yield of photosystem II ($\phi$$_{psII}$) to $C_o$ largely paralleled
Figure 1. Irradiance and CO₂ response of net photosynthesis rates in rca-2, rwt43 and spsa1 (a,b), aba2-1 and C24 (c,d) and npq1-2 and npq4-1 (e,f). Col-0 is included in each panel for ease of comparison. In (d), supply lines between Ca = 500 and the corresponding Ci response curve of An are shown to emphasize stomatal effects of aba2-1, C24 and Col-0 on Ci. Averages ± SEM, n = 5–15.

Table 1. Parameters derived from Ci response curves of An. Vₘₐₓ, maximum caboxylation rate by Rubisco (μmol CO₂ m⁻² s⁻¹); J_max, maximum rate of electron transport in the absence of regulation (μmol electrons m⁻² s⁻¹); TPU, maximum rate of triose phosphate utilisation (μmol CO₂ m⁻² s⁻¹). The root mean squared error (RMSE, μmol CO₂ m⁻² s⁻¹) of the differences between measurement and model during curve fitting is shown as an estimation of the overall goodness of fit. Averages ± SEM, n = 5–15. Stars within columns denote significance levels compared to Col-0: ***P < 0.0001, **P < 0.01, *P < 0.05. Absence of stars denotes lack of significant difference with Col-0 (P > 0.05).
that of An, with the exception that \( \phi_{\text{PSII}} \) decreased at high \( C_i \) in many genotypes (except \( rca-2 \) and \( npq4-1 \); see Supplementary Fig. 1). This decrease in \( \phi_{\text{PSII}} \) was most marked, and started at a lower \( C_i \), in \( spsa1 \) (Supplementary Fig. 1a).

Larger Rubisco activation state and \( g_s \), accelerate photosynthetic induction, while lower NPQ does not. Next, we characterised the dynamic behaviour of leaf gas exchange by inducing photosynthesis in dark-adapted leaves using a stepwise increase to saturating irradiance (1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). Rates of photosynthetic induction were initially similar between all genotypes (except \( rwt43 \)) until \(~60\%\) induction was reached (Fig. 2). \( rwt43 \) reached 50\% of photosynthetic induction (tA50) significantly faster than Col-0 (Table 2). Induction remained faster in \( rwt43 \) until it reached \(~80\%\) (Fig. 2a). In \( rca-2 \), the rate of induction slowed after 60\% completion and then increased in a nearly linear fashion rather than the more exponential increase shown by all other genotypes (Fig. 2a). This increased the time to reach 90\% of photosynthetic induction (tA90) by \(~10\) minutes compared to Col-0. \( spsa1 \) showed slightly slower induction rates (Fig. 2a), increasing tA90 by \(~5\) min compared to Col-0. \( aba2-1 \) exhibited faster induction, halving the tA90 of Col-0, while induction in C24 was identical to that of Col-0 (Fig. 2b). Induction in \( npq1-2 \) and \( npq4-1 \) was identical to Col-0 (Fig. 2c).

To explain the differences between genotypes affecting Rubisco activation and \( g_s \), we looked at the time courses of \( C_i \), diffusional limitation and biochemical limitation. While \( C_i \) in Col-0 and \( rwt43 \) dropped by \(~130\) ppm within 10 minutes and then increased by \(30–40\) ppm following stomatal opening, in \( rca-2 \) it never dropped below its final value (Fig. 3a). Diffusional limitation reached its maximum within \(~10\) minutes in Col-0 and \( rwt43 \) and then relaxed, while in \( rca-2 \) its increase was much slower and levelled off after \(~30\) minutes (Fig. 3c). Biochemical

**Figure 2.** Photosynthetic induction after a step increase in irradiance from 0 to 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) in \( rca-2 \), \( rwt43 \) and \( spsa1 \) (a), \( aba2-1 \) and C24 (b) and \( npq1-2 \) and \( npq4-1 \) (c). Col-0 is included in each panel for ease of comparison. Averages ± SEM, \( n = 5–15 \).
limitation during induction relaxed almost completely within ~10 minutes in Col-0 and rwt43, while in rca-2 it was generally greater and the same extent of relaxation took ~40 minutes (Fig. 3e). Comparing Col-0 and C24, the responses of Ci were almost indistinguishable, while in aba2-1 the initial decrease in Ci was smaller, ranging from

| Genotype | 0 → 1000μmol m⁻² s⁻¹ | 70 → 800μmol m⁻² s⁻¹ | 130 → 600μmol m⁻² s⁻¹ |
|----------|-----------------------|-----------------------|-----------------------|
| tA50     | tA90                  | tA50                  | tA90                  |
| Col-0    | 1.6 ± 0.1             | 14.7 ± 1.2            | 1.3 ± 0.1             | 10.2 ± 1.1            | 0.6 ± 0.0             | 9.0 ± 2.2             |
| rca-2    | 1.5 ± 0.2             | 25.5 ± 1.5***         | 6.3 ± 0.4***          | 30.9 ± 2.0***         | 4.0 ± 0.7***          | 29.8 ± 1.7***         |
| rwt43    | 1.2 ± 0.1**           | 14.2 ± 2.6            | 0.5 ± 0.0***          | 16.2 ± 6.1            | 0.3 ± 0.0**           | 18.8 ± 6.1            |
| spa1     | 1.6 ± 0.1             | 19.5 ± 1.3*           | 1.3 ± 0.1             | 14.1 ± 7.2            | 0.6 ± 0.1             | 13.7 ± 6.9            |
| aba2-1   | 1.4 ± 0.1             | 7.3 ± 0.5**           | 1.3 ± 0.1             | 7.7 ± 2.6             | 0.8 ± 0.1             | 15.1 ± 5.8            |
| C24      | 1.9 ± 0.1             | 15.0 ± 3.2            | 1.7 ± 0.3*            | 13.3 ± 2.7            | 0.9 ± 0.2*            | 29.4 ± 5.1***         |
| npq1-2   | 1.4 ± 0.1             | 11.7 ± 1.7            | 1.3 ± 0.1             | 10.7 ± 2.9            | 0.7 ± 0.0             | 14.6 ± 8.6            |
| npq4-1   | 1.5 ± 0.1             | 14.8 ± 2.6            | 1.1 ± 0.1             | 6.1 ± 0.7             | 0.6 ± 0.0             | 15.3 ± 11.0           |

Table 2. Time (minutes) to reach 50 and 90% of steady-state photosynthesis rates (tA50, tA90) after step increases in irradiance. Averages ± SEM, n = 5–15. Stars within columns denote significance levels compared to Col-0: ***P < 0.0001, **P < 0.01, *P < 0.05. Absence of stars denotes lack of significant difference with Col-0 (P > 0.05).
50–60% of that found in Col-0 (Fig. 3b). Buildup and relaxation of diffusional limitation were much smaller in aba2-1 (Fig. 3d), while relaxation of biochemical limitation was similar between Col-0, aba2-1 and C24 (Fig. 3f).

Next to the dark-light transition discussed above, we also exposed leaves that had been adapted to low irradiance (hereafter: background irradiance) to stepwise increases in irradiance, namely 70 → 800 and 130 → 600 μmol m⁻² s⁻¹. The responses of An to these increases were qualitatively similar to those seen after the dark-light transition (Supplementary Fig. 2).

rwt43 exhibited a faster increase, and rca-2 a much slower increase than Col-0 (Supplementary Fig. 2a,b). This reduced tA50, but not tA90, in rwt43, while tA50 and tA90 in rca-2 were larger than Col-0 (Table 2). C24 tended to increase photosynthesis more slowly compared to Col-0 (Supplementary Fig. 2c,d), leading to a larger tA50 after the 70 → 800 μmol m⁻² s⁻¹ step increase and larger tA50 and tA90 after the 130 → 600 μmol m⁻² s⁻¹ step increase. Assimilation responses in NPQ and SPS mutants to those intermediate irradiance increases were similar to Col-0.

Apart from gas exchange dynamics, we also characterised changes in electron transport parameters after the stepwise 0–1000 μmol m⁻² s⁻¹ transition. Changes in φPSII largely paralleled those of Aₙ (Fig. 4). In rwt43, the increase in φPSII was slightly faster than in Col-0, while in rca-2, it was slower and steady-state φPSII was lower (Fig. 4a), paralleling its lower steady-state Aₙ (Fig. 1a). Despite slightly larger φPSII throughout induction in spsa1, final values were not significantly different from Col-0 (P = 0.09, Fig. 4a). aba2-1 showed increased steady-state φPSII levels, while in C24 they were reduced compared to Col-0 (Fig. 4c), similar to the differences in steady-state assimilation (Fig. 1c). In npq4-1, φPSII was slightly lower during induction than in npq1-2 and Col-0 (npq1-2 had similar φPSII trends and values during induction as Col-0; Fig. 4e). NPQ in rca-2 increased more quickly to its steady-state level, which was larger than that of Col-0, spsa1 and rwt43 (Fig. 4b). NPQ in aba2-1 was lower than in Col-0 and C24 (which were not significantly different from each other, Fig. 4d). As expected, npq1-2 and npq4-1 developed much lower NPQ levels than Col-0, and NPQ buildup was slower compared to Col-0, but similar in
both npq1-2 and npq4-1 (Fig. 4f). Dark-adapted Fv/Fm was 0.805 ± 0.002 (Avg ± standard error of the mean, SEM) in Col-0. In rca-2, C24 and npq4-1, Fv/Fm was marginally, but significantly, smaller, possibly due to photoinhibition that was not completely removed by dark adaptation. In spsa1, it was slightly but significantly higher than in Col-0 (Supplementary Fig. 3).

Isoform, amount and initial activation state of Rca affect the rate of Rubisco activation. The apparent time constants of Rubisco activation ($\tau_R$, the time to reach 63% of total change in Rubisco activation state), decreased with increasing background irradiance (Fig. 5). Genotypes differing in gs, NPQ and SPS did not differ from Col-0 in $\tau_R$. However, $\tau_R$ tended to be 17–28% larger in spsa1 than in Col-0; P-values ranged from 0.07 to 0.09. Of the genotypes affecting Rca regulation, rca-2 exhibited the biggest differences in $\tau_R$, both compared with Col-0 (P < 0.001 in all cases) and between background irradiances, with a $\tau_R$ of ~22 minutes in dark-adapted leaves decreasing to ~4 minutes in leaves adapted to an irradiance of 130 $\mu$mol m$^{-2}$ s$^{-1}$ (Fig. 5a). In rwt43, $\tau_R$ of dark-adapted leaves (2.3 min) was not significantly different to that of Col-0 (3.0 min; P = 0.08), but was significantly (P < 0.001) smaller at 70 and 130 $\mu$mol m$^{-2}$ s$^{-1}$ background irradiance (Fig. 5b).

Increases in initial gs, up to a threshold value accelerate photosynthetic induction. Before and after stepwise increases in irradiance, gs was considerably higher in aba2-1 than in Col-0 and C24 (Supplementary Fig. 4). In dark-adapted leaves of Col-0 and C24, gs was similar, but in leaves adapted to 70 or 130 $\mu$mol m$^{-2}$ s$^{-1}$, it was almost twice as high in Col-0 compared to C24. This spread in gs was used to explore the threshold between a limiting and a non-limiting initial gs for the subsequent rates of An increase. For example, after the 0 → 1000 $\mu$mol m$^{-2}$ s$^{-1}$ increase, tA90 was lower in plants with initially higher gs up to ~0.13 mol m$^{-2}$ s$^{-1}$, but above 0.13 mol m$^{-2}$ s$^{-1}$ there was no further decrease in tA90 (Fig. 6). This shows that an initial gs > 0.13 mol m$^{-2}$ s$^{-1}$ was non-limiting in this case. We also looked at various time points (tA10, tA20, etc.) after different low-to-high irradiance transitions (i.e. 0 → 1000, 70 → 800 and 130 → 600 $\mu$mol m$^{-2}$ s$^{-1}$) and found that the threshold between limiting and non-limiting initial gs was between 0.09 and 0.17 mol m$^{-2}$ s$^{-1}$, with no discernible trend between time points or background irradiance levels.

Apart from the effect of initial gs on the rate of $A_n$ increase, we also analysed the effects of gs increase after stepwise increases in irradiance (Supplementary Fig. 4). In C24 and Col-0, the increase in gs after the 0 → 1000 $\mu$mol m$^{-2}$ s$^{-1}$ increase (until 60 minutes after the start of illumination) and tA80 correlated positively (Supplementary Fig. 5). Because initial gs in aba2-1 was high, it was non-limiting to rates of increase in photosynthesis after irradiance increases, and stomatal opening did not correlate with tA80 (data not shown).

Figure 5. Apparent time constant of Rubisco activation in rca-2 (a) and rwt43 (b), compared to Col-0. Note the different scales of Y-axes in (a,b). Averages ± SEM, n = 5–15. Bars in (b) at 30 $\mu$mol m$^{-2}$ s$^{-1}$ background irradiance included from Carmo-Silva and Salvucci42. Stars denote significance levels of single genotypes compared to Col-0: ***P < 0.001.
Lower NPQ and SPS do not do increase transient photosynthesis after a decrease in irradiance.

After step decreases in irradiance (600 → 200, 800 → 130 μmol m⁻² s⁻¹), relative changes in A₀ were similar for all genotypes (Supplementary Fig. 6), and there were no significant differences in either post-illumination CO₂ fixation or the post-illumination CO₂ burst, including the NPQ mutants and spsa1 (Supplementary Fig. 7).

Discussion

Making use of the genetic diversity available for A. thaliana, we explored several possible physiological limitations of dynamic photosynthesis. This analysis revealed that altered Rubisco activation kinetics or stomatal conductance affect photosynthesis in a dynamic irradiance environment greatly, while alterations in non-photochemical quenching or sucrose synthesis do not.

Changes affecting Rca concentration (rca-2) or regulation (rwt43) had strong effects on dynamic photosynthesis. The observed effects were likely caused by different kinetics of Rubisco activation, as the initial increase in assimilation after dark-light transitions (first minute in Fig. 2a) was similar between genotypes, implying a similar limitation due to activation of RuBP regeneration (Sassenrath-Cole and Pearcy22 provided biochemical evidence for this). Furthermore, these genotypes had similar gₛ (Supplementary Fig. 8). Lower steady-state irradiance and CO₂ responses in rca-2 may have been caused by a reduced steady-state activation of Rubisco20. Based on the dependency between maximum Rubisco activation state and Rca concentration reported by Mott and Woodrow30 and our estimation of Vₘₐₓ for rca-2 (Table 1), we estimate that rca-2 contains ~22% of wildtype Rca levels (Supplementary text 1). The effects on the rate of Rubisco activation of such low Rca content are apparent. In antisense or overexpressors of Rca in rice, a positive linear relationship between Rca concentration and the rate of photosynthetic induction was shown for various temperatures41, demonstrating the role of Rca concentration in controlling dynamic photosynthesis. Intriguingly, in our study τₚ decreased with background irradiance (Fig. 5). While this decrease was linear in Col-0, it resembled a negative exponential in rwt43. This is in agreement with data of Carmo-Silva and Salvucci42 (Fig. 5b). Previous studies have shown that Rubisco activation in Col-0 increased linearly with irradiance42–44, while in rwt43, Rubisco activation state did not change with increasing irradiance42; it was similar to Col-0 in dark-adapted leaves, but close to full activation in low irradiance19,42,44. Most likely differences in the activation state of Rca, rather than that of Rubisco, caused τₚ to decrease with background irradiance. Rca activity increased linearly between 0 and 300 μmol m⁻² s⁻¹ in intact spinach leaves45, and should be high in rwt43 except in darkness (see above).

Compared to natural fluctuations in irradiance, stomata open and close slowly46. Low initial gₛ can become a limitation to carbon fixation after a step change in irradiance2, because of comparably rapid activation of RuBP regeneration and Rubisco. The peak of this limitation is typically reached within ~10 minutes due to Rubisco activation without similarly large increases in gₛ, after which it relaxes due to stomatal opening (Fig. 3d). We note that the index of diffusional limitation should be refined with respect to changes in Rubisco activation during photosynthetic induction, as well as possible changes in mesophyll conductance (gₚ,m) during transients. With respect to gₛ, contrasting responses to irradiance have been reported (cf. refs 47 and 48); we therefore refrain from speculations on how it may have changed in our measurements but note that it may have affected the index of diffusional limitation. Nevertheless, we believe that diffusional limitation provides a useful qualitative tool to analyse the differences between the genotypes affecting Rubisco activation kinetics and gₛ.

The mutant with high initial gₛ (aba2-1) did not show such large differences in stomatal opening (i.e. difference between initial and final gₛ; Supplementary Fig. 4), but still had much higher rates of A₀, increases when irradiance was raised. Therefore, we argue that increasing the initial gₛ is a simpler route to increasing dynamic photosynthesis than is increasing the rate of stomatal opening. Stomatal closure in low irradiance is an adaptive response to changing water supply and logical under non-irrigated field conditions, however for crops in well-watered situations, increasing gₛ at the expense of water use may be a reasonable target to increase rates of dynamic photosynthesis. Also, the threshold between limiting and non-limiting gₛ for rates of photosynthesis increase could be used as a phenotypic marker for breeding of cultivars with non-limiting gₛ, in fluctuating irradiance. In our analysis, this threshold proved to be consistent, independent of the time point after stepwise increases in irradiance and level of background irradiance. Previous findings indicate that this threshold shows no diurnal variation26.

Figure 6. Relationship between initial gₛ and the time to reach 90% of final photosynthesis rates after a step increase in irradiance (0–1000 μmol m⁻² s⁻¹) in single replicates of Col-0, aba2-1 and C24.
and that it is unchanged by water stress\textsuperscript{35} or growth light conditions\textsuperscript{36}. An open question that remains is whether the threshold is species-specific\textsuperscript{32} or not\textsuperscript{36}. It is likely that a high initial g\textsubscript{s} correlates with constitutively high g\textsubscript{i} (i.e. stomata are more open and less sensitive to changes in irradiance), and faster responses of A\textsubscript{n} to an increasing irradiance could be reached at the expense of lower intrinsic water use efficiency. Rapid screening for high g\textsubscript{i} could be achieved by thermal imaging\textsuperscript{39}.

In Col-0, rates of NPQ buildup after a dark-light transition were similar to those seen in previous studies\textsuperscript{33,52}, while mutants npq\texttextsuperscript{1-2} (lacking violaxanthin de-epoxidase\textsuperscript{31}) and npq\texttextsuperscript{4-1} (lacking PsbS\textsuperscript{30}) exhibited a much lower buildup of NPQ. However, they showed negligible differences in gas exchange to Col-0, neither in their steady-state responses to irradiance and CO\textsubscript{2} (Fig. 1e,f) nor in their responses to step increases in irradiance (Fig. 2c, Supplementary Fig. 2c,f). Similar to our findings, reduced PsbS content in transgenic rice plants strongly reduced NPQ but had limited effects on carbon gain during a 5-min induction period\textsuperscript{53}. In contrast, overexpressors with 2–4 fold increases in PsbS showed ~15% lower A\textsubscript{n} during induction, demonstrating that increased energy dissipation can have adverse effects on assimilation\textsuperscript{25}. Two antisense mutants with reduced thylakoid membrane K\textsubscript{+} flux capacities showed less rapid relaxation of NPQ after irradiance decreases, reducing electron transport and assimilation\textsuperscript{24}. Our data revealed no differences between npq\texttextsuperscript{1-2}, npq\texttextsuperscript{4-1} and Col-0 with respect to post-illumination CO\textsubscript{2} fixation (Supplementary Fig. 7), and therefore show that unlike the rate of NPQ relaxation\textsuperscript{22,23}, an initially low level of NPQ does not increase carbon gain directly after decreases in irradiance.

Irradiance-dependent activation of SPS is genotype-specific, and A. \textit{thaliana} belongs to a group of species with low light/dark modulation of the enzyme\textsuperscript{31}. This suggests that in the wildtype, SPS activity does not limit photosynthetic induction—however, in a plant with strongly reduced SPS concentration it might. We tested this possibility in the T-DNA mutant \textit{spsa}1, which has a 80% lower maximum SPS activity than Col-0\textsuperscript{28}. Similar to our findings, Sun et al.\textsuperscript{25} found no photosynthetic differences between \textit{spsa}1 and Col-0, except for a strong reduction in CO\textsubscript{2}-saturated A\textsubscript{n} (−23%). Importantly, the decrease in NPQ has an adverse effect on photosynthetic responses to fluctuating irradiance. The only significant difference was a longer time to reach 90% of full induction after dark-light transitions (Table 2). However, no such differences were observed in transitions from low to higher irradiance. \textit{spsa}1 would probably show decreased rates of dynamic photosynthesis in elevated CO\textsubscript{2} concentrations. Furthermore, it may be that the absence of a measurable effect of \textit{spsa}1 on the post-illumination CO\textsubscript{2} burst, which is partly affected by the rate of sucrose synthesis\textsuperscript{31}, was masked by the photorespiratory portion of the CO\textsubscript{2} burst, which is most pronounced in C\textsubscript{3} plants\textsuperscript{5}. Also, reduced levels of SPS in species that exhibit strong light/dark modulation of SPS (e.g. barley, maize, spinach and sugarbeet\textsuperscript{39}) would probably have a stronger negative effect on photosynthetic induction than shown here for \textit{A. thaliana}.

The relationship between $\Phi_{\text{PSII}}$ and C\textsubscript{i} in C\textsubscript{3} photosynthesis contains three phases: When A\textsubscript{n} is (a) limited by Rubisco, $\Phi_{\text{PSII}}$ increases with C\textsubscript{i}; when A\textsubscript{n} is (b) limited by RuBP regeneration, $\Phi_{\text{PSII}}$ is constant with increases in C\textsubscript{i} and when A\textsubscript{n} is (c) limited by TPU, $\Phi_{\text{PSII}}$ decreases with increasing C\textsubscript{i}\textsuperscript{54,55}. Most genotypes in our study did not show the plateau in $\Phi_{\text{PSII}}$ that would signify a phase of RuBP regeneration limitation, with $\Phi_{\text{PSII}}$ showing an extreme form of that behaviour (Supplementary Fig. 1). This suggests that (a) TPU occurs at a lower C\textsubscript{i} than visible from gas exchange, (b) different limitations occur simultaneously within different layers of the leaf, (c) changes in the rate of cyclic electron transport around photosystem I and/or strength of alternative electron sinks or (d) with increasing C\textsubscript{i} during the phase of limitation by RuBP regeneration photosynthetic electron transport is sometimes restricted, and $\Phi_{\text{PSII}}$ is reduced, due to the increased inhibition of starch synthesis following the inhibition of phosphoglucoisomerase by phosphoglycerate\textsuperscript{26}. However, these results have to be interpreted with caution because the number of data points between the end of Rubisco limitation and the onset of TPU was limited and more data may lead to different conclusions.

In conclusion, in \textit{A. thaliana}, the presence of the redox-regulated $\alpha$-isoform of Rca in the wildtype, and wildtype levels of g\textsubscript{s} are limiting for dynamic photosynthesis. Furthermore, reductions in Rca strongly decrease (dynamic) photosynthesis. We also show that wildtype levels of NPQ and SPS are not limiting in \textit{A. thaliana}. This suggests Rca and g, as targets for improvement of photosynthesis in fluctuating irradiance.

**Methods**

**Plant material.** Seeds of npq\texttextsuperscript{4-1}, \textit{spsa}1 (SALK\textsubscript{148643C}) and rca\texttextsuperscript{2-1} (SALK\textsubscript{003204C}) were obtained from NASC (University of Nottingham, Loughborough, UK\textsuperscript{57}). C24 (CS76106) was obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, USA). Seeds of Col-0 and aba2\texttextsuperscript{1-1} were obtained from Corrie Hanhart ( Wageningen University, the Netherlands), npq\texttextsuperscript{1-2} was obtained from Dr. Shizue Matsubara (Forschungszentrum Jülich, Germany) and rwt\texttextsuperscript{63} was obtained from Dr. Elizabete Carmo-Silva ( Rothamsted Research, UK).

**Growth conditions.** Plants were grown in 0.37 L pots using soil with a 4:1 peat:perlite mixture. Pots were placed on irrigation mats, and mats were saturated daily to full capacity. Plants were fertilized weekly using a nutrient solution especially developed for Arabidopsis\textsuperscript{38}. To inhibit algal growth, the soil was covered with black plastic film. Plants were grown in a growth chamber in short day–long night conditions (8 hours of light) to delay flowering\textsuperscript{39} and thus ensure that leaves were large enough for gas-exchange measurements. Irradiance was 172 ± 4 µmol m\textsuperscript{-2} s\textsuperscript{-1} as supplied by LED lights (GreenPower LED production module deep red/white 120; Philips, Eindhoven, the Netherlands; Supplementary Fig. 9). Temperature was 23/18°C (day/night) and relative humidity was 70%. Mutants lacking ABA (aba2\texttextsuperscript{1-1}) were spray-dried with an aqueous solution containing 10 µmol mol\textsuperscript{-1} ABA (Sigma, St. Louis, U.S.A.) when plants were 2, 4 and 6 weeks old. This increases Rosette growth compared to untreated aba2\texttextsuperscript{1-1} plants (data not shown). There was a period of 15 days between the last application of ABA and the first measurements on aba2\texttextsuperscript{1-1} plants.

Single genotypes were grown sequentially (approx. one batch per week). Five plants per batch were used for measurements. To monitor the quality of the growth system over time, Col-0 was grown in three batches, each...
The growth system produced very reproducible photosynthetic phenotypes of Col-0 (Supplementary Fig. 10). Measurements. Measurements were performed using the LI-6400 portable photosynthesis system (Li-Cor Biosciences, Lincoln, Nebraska, USA) equipped with the leaf chamber fluorometer (Part No. 6400-40) on single leaves of plants that were 6–8 weeks old. Leaves large enough to cover the leaf chamber gasket (area: 2 cm², diameter: 1.6 cm) were used. Conditions in the cuvette were as follows: 23°C air temperature, 70% relative humidity, 90/10% red/blue light mixture and 500 μmol s⁻¹ air flow rate. The choice of flow rate was a compromise between getting a fast time response of the measuring system (necessary in dynamic gas exchange studies), and the difference in CO₂ concentration between sample and reference air stream. Except for the CO₂-response curves, the external CO₂ mole fraction was kept at 400 ppm. The oxygen mole fraction was always 21%.

Stepwise increases in irradiance. Leaves were adapted to several background irradiances (0, 70 or 130 μmol m⁻² s⁻¹) for 30–60 minutes (until An and gs had reached a steady state), and then exposed to single-step increases in irradiance, namely 0 → 1000, 70 → 800 and 130 → 600 μmol m⁻² s⁻¹. These intensities were chosen, after preliminary irradiance-response curves on Col-0 had shown that all but the highest (1000 μmol m⁻² s⁻¹) intensity were in the sub-saturating range (Supplementary Fig. 11). Gas exchange was logged nominally every second. Logging was stopped when gₚ reached a new steady state (this was assessed visually, and took a minimum of 30 minutes after the step increase), or 60 minutes after switching to 1000 μmol m⁻² s⁻¹. Before and after the 0 → 1000 μmol m⁻² s⁻¹ increase, φₚₛₚₛ and NPQ were measured, using a measuring beam intensity of ~1 μmol m⁻² s⁻¹ and a saturating pulse of ~7600 μmol m⁻² s⁻¹ intensity and 1 s duration. In preliminary measurements on Col-0, the saturating pulse was sufficient to saturate Fₚₚₛₛ in leaves adapted to 1000 μmol m⁻² s⁻¹ (assessed following the manufacturer’s recommendations for calibrating the saturating pulse: Fₚₚₛₛ was not increased when using saturating pulses of intensity higher than 7600 μmol m⁻² s⁻¹). The Fₚ and Fₚₛₛ relative fluorescence yields were measured in dark-adapted leaves. After the increase in irradiance, the Fₚₛₛ relative fluorescence yield was measured every minute for the first ten minutes, and every two minutes thereafter. The regular application of saturating flashes transiently increased the leaf temperature by 0.4–0.7°C across genotypes (temperature traces of Col-0 are representative of all genotypes, Supplementary Fig. 12). Also, our data (Kaiser et al., unpublished) indicate that the regular application of saturating flashes of similar intensity and frequency in tomato (Lycopersicon esculentum) had no effects on leaf gas exchange during photosynthetic induction. The steady-state relative fluorescence yield, Fₚₛₛ, was measured continuously. Dark-adapted Fₛₛ/Fₚₛₛ and NPQ were calculated as Fₛₛ/Fₚₛₛ = (Fₚₛₛ/Fₚₛₛ)/Fₚₛₛ and NPQ = (Fₚₛₛ/Fₚₛₛ)/Fₚₛₛ, respectively.

During transients, gₛₛ and mitochondrial respiration (Rₛₛ) were assumed to be constant because, to our knowledge, changes in gₛₛ and Rₛₛ have never been assessed for irradiance transients. Rₛₛ in the light was considered similar to genotype-specific steady-state respiration in the dark; this assumption is supported by measurements on several species. For gₛₛ, a value of 0.2 μmol m⁻² s⁻¹ was assumed for all genotypes, which is an average of three values determined on Col-0 of comparable photosynthetic capacity.

The time to reach 50 and 90% (i.e. t₅₀ and t₉₀) of steady-state An was calculated for each irradiance increase. To increase robustness of these indices to experimental noise and outliers, time series were smoothed using a local polynomial regression with a span of 5%. This means that, for each point in the time series, a polynomial of degree two was fitted using weighted least squares to a data window of size equal to 5% of the total size of the time series. To calculate several parameters, An was corrected for transient changes in chloroplast CO₂ concentration (Cₐ). For diffusional limitation, Aₙ was multiplied by the relative rate by which An would increase if Cₐ during induction was equal to ambient CO₂ concentration, Cₐ (Anₙₚₚₛₛ(Cₐ)):

\[ Aₙₚₚₛₛ = Aₙ * \frac{f(Cₐ)}{f(Cₐ)} \]  

(1)

Where f(Cₐ) is the steady-state value of Aₙ at Cₐ (i.e. at 400 ppm), and f(Cₐ) is the steady-state value of Aₙ at Cₐ. The relative effects of Cₐ on Aₙ were taken from steady-state Aₙ/Cₐ response curves by fitting local polynomial regressions (LOESS) in the range 50–500 ppm (Supplementary Fig. 13). Diffusional limitation was then determined as:

\[ \text{Diffusional limitation} = \frac{Aₙₚₚₛₛ - Aₙ}{Aₙₚₚₛₛ - Aₙₖ} \times 100 \]  

(2)

Where Aₙₚₚₛₛ is the steady-state value of Aₙ at Cₐ and Aₙₖ is the initial steady-state rate of Aₙ. Diffusional limitation is therefore a combination of possible limitations due to gₛₛ and gₛₛ during induction and in the steady state (i.e. it does not decrease to 0% at the end of the time course). For biochemical limitation and tₕ, Aₙ was multiplied (Aₙₚₚₛₛ(Cₐ)) by the relative rate by which Aₙ would increase if transient Cₐ was equal to final, steady-state Cₐ (Cₐₚₚₛₛ), following Woodrow and Mott:

\[ Aₙₚₚₛₛ = Aₙ * \frac{f(Cₐ)}{f(Cₐ)} \]  

(3)

Where f(Cₐ) is the solution for Aₙ at Cₐ. Biochemical limitation was calculated after Allen and Pearcy.
Biochemical limitation = \frac{A_{nf} - A_{nf}^*}{A_{nf} - A_{ni}} \cdot 100 \quad (4)

Throughout induction, biochemical limitation decreases from 100 to 0%, and therefore indicates the additional limitation imposed on An due to incomplete activation of several enzymes. Biochemical and diffusional limitations do not sum up to 100%, and are distinct. The apparent time constant of Rubisco activation (\tau_R) was calculated after Woodrow and Mott \cite{55}:

\tau_R = \frac{\Delta \text{time}}{\Delta \ln (A_{nf} - A_{nf}^*)} \quad (5)

The range of timepoints (\Delta \text{time}) for calculating \tau_R differed between background irradiances (Supplementary Fig. 14), and in some cases between genotypes. This was due to differences in the rate of change of photosynthesis, and included 120 data points in the case of 0 \rightarrow 1000 \mu mol m^{-2} s^{-1} (all genotypes) and 40 (for \textit{rwt43}) or 60 (all other genotypes) in the case of 70 \rightarrow 800 and 130 \rightarrow 600 \mu mol m^{-2} s^{-1}. These ranges were selected by visual inspection. The average root mean squared error of the linear fits was 1.2 \mu mol m^{-2} s^{-1} (range: 1.0–3.0 \mu mol m^{-2} s^{-1}).

Stepwise decreases in irradiance. Irradiance was decreased in the following steps: 800 \rightarrow 130 and 600 \rightarrow 200 \mu mol m^{-2} s^{-1}. From the CO2 exchange data, post-illumination CO2 fixation \cite{39} and post-illumination CO2 burst \cite{40} were quantified. The former implies that photosynthesis is above the final steady-state value during the transient, while the latter implies a lower assimilation rate than at steady state. Values were estimated by integrating the difference between time series of photosynthesis and the final steady-state value \cite{66}.

Irradiance response curves. When An was at a steady state, i.e. before step changes in irradiance or at the end of a measurement sequence, 120 data points were used to extract average An at a given irradiance. The resulting values were used to construct steady-state irradiance response curves.

CO2 response curves. Leaves were adapted to 1000 \mu mol m^{-2} s^{-1} for ~30 min and 500 ppm C4. C4 was then decreased stepwise until 50 ppm, each step taking 2–3 minutes. Thereafter, C4 was raised to 500 ppm, and after waiting for ~15 minutes, leaves were exposed to stepwise increases in C4 until 1500 ppm, each step taking ~4 minutes. Values were logged every 5 s and the last 60 s of every CO2 step used to calculate average \text{Ci} and \text{An}.

Statistical analysis. Each genotype was compared to Col-0 using a Student’s \textit{t}-test (Microsoft Excel, function \textit{t}-test, assuming 2-tailed distribution and two-sample equal variance).

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