

# The time course of acclimation to the stress of triose phosphate use limitation

Alan M. McClain<sup>1,2,3</sup>  | Jeffrey A. Cruz<sup>1</sup> | David M. Kramer<sup>1,2</sup>  | Thomas D. Sharkey<sup>1,2,4</sup> 

<sup>1</sup>Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan, USA

<sup>2</sup>Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan, USA

<sup>3</sup>Plant Biotechnology for Health and Sustainability, Michigan State University, East Lansing, Michigan, USA

<sup>4</sup>Plant Resilience Institute, Michigan State University, East Lansing, Michigan, USA

## Correspondence

Thomas D. Sharkey, Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA.  
Email: [tsharkey@msu.edu](mailto:tsharkey@msu.edu)

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## Abstract

Triose phosphate utilisation (TPU) limits the maximum rate at which plants can photosynthesise. However, TPU is almost never found to be limiting photosynthesis under ambient conditions for plants. This, along with previous results showing adaptability of TPU at low temperature, suggest that TPU capacity is regulated to be just above the photosynthetic rate achievable under the prevailing conditions. A set of experiments were performed to study the adaptability of TPU capacity when plants are acclimated to elevated CO<sub>2</sub> concentrations. Plants held at 1500 ppm CO<sub>2</sub> were initially TPU limited. After 30 h they no longer exhibited TPU limitations but they did not elevate their TPU capacity. Instead, the maximum rates of carboxylation and electron transport declined. A timecourse of regulatory responses was established. A step increase of CO<sub>2</sub> first caused PSI to be oxidised but after 40 s both PSI and PSII had excess electrons as a result of acceptor-side limitations. Electron flow to PSI slowed and the proton motive force increased. Eventually, non-photochemical quenching reduced electron flow sufficiently to balance the TPU limitation. Over several minutes rubisco deactivated contributing to regulation of metabolism to overcome the TPU limitation.

## KEYWORDS

energy-dependent exciton quenching, photosynthetic control, Rubisco activation, thylakoid ATP synthase, triose phosphate use (TPU)

## 1 | INTRODUCTION

Photosynthesis, as measured by gas exchange, is typically assessed by the three canonical biochemical limitations of photosynthesis: the rubisco limitation, where carbon dioxide uptake is modelled assuming ribulose 1,5-bisphosphate (RuBP)-saturated rubisco kinetics; the RuBP regeneration limitation, where carbon dioxide uptake is modelled assuming a fixed rate of RuBP use as allowed by the production of electron transport products, ATP and NADPH; and

the triose phosphate utilisation (TPU) limitation, where carbon dioxide uptake is modelled as the rate of production of end products, freeing inorganic phosphate from organic phosphates (McClain & Sharkey, 2019). The TPU limitation is not always observed and whether it should be included in models of global photosynthesis has been debated (Lombardozzi et al., 2018; McClain & Sharkey, 2019; Rogers et al., 2020).

The TPU limitation is unique among the three biochemical limitations in that it is limited by processes downstream of the

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Calvin-Benson cycle. Rather than running carbon fixation and electron transport as efficiently as possible, regulatory mechanisms are engaged to slow down the rate of carbon assimilation ( $A$ ) so as not to outpace the rate of end-product synthesis. Energy-dependent quenching ( $q_E$ ) is activated (Sharkey et al., 1988) by elevated  $\Delta pH$  across the thylakoid membrane, one component of proton-motive force (PMF) (Kramer & Crofts, 1996). The elevated  $\Delta pH$  results from kinetic and thermodynamic restrictions on the ATPase due to lowered levels of available inorganic phosphate (Sharkey & Vanderveer, 1989). In addition, rubisco activation state decreases (Sharkey et al., 1986a; Socias et al., 1993), which may alleviate pressure on phosphate pools by limiting the maximum rate that carbon can be added to the organic phosphate pool. Because TPU limitation restricts the rate of photosynthesis rather than the availability of light, there is a potential for photodamage unless regulatory mechanisms are engaged (Li et al., 2002; Pammenter et al., 1993; Powles, 1984).

These regulatory mechanisms are the only aspects of TPU limitation typically observed in steady-state gas exchange. While TPU limitation results in and can be assessed through gas exchange as  $O_2$ - and  $CO_2$ -insensitive photosynthesis (Sharkey, 1985) or reverse sensitivity to  $O_2$  (Viil et al., 1977) or  $CO_2$  (Jolliffe & Tregunna, 1973), it is easier to assess by the decline in electron transport rate associated with  $q_E$  when  $CO_2$  is increased or  $O_2$  is decreased. The appearance of transient effects on photosynthesis associated with TPU limitation (Ogawa, 1982; Walker et al., 1983) lead us to believe that, in the steady state, the rate of photosynthesis is not set by TPU, but instead, the rate is set by regulatory mechanisms that match the rates of carbon input to and carbon output from the organic phosphate pool.

TPU capacity does not require many resources. The nitrogen required for rubisco and photosynthetic electron transport far exceed those required for TPU and subsequent end product synthesis (Evans & Clarke, 2019). When TPU occurs, rubisco is deactivated and  $q_E$  is increased reducing the efficiency of nitrogen use in both carbon metabolism and electron transport. Entering TPU limitation forces deactivation of systems which use much more nitrogen, an ideal plant would never experience TPU limitation under physiological conditions. However, TPU limitation is commonly seen when the photosynthetic rate is only a few percent higher than what the plant experiences in ambient conditions (Yang et al., 2016). There are a few possible reasons why excess TPU capacity would be detrimental. A precise balance of phosphate flux could control stromal inorganic phosphate concentration, affecting the partitioning of carbon into starch (Escobar-Gutierrez & Gaudillere, 1997; Preiss, 1982). If TPU capacity were in excess, it could also limit the ability to build up a PMF across the thylakoid membrane because there would be plentiful phosphate available to the ATPase, preventing any kinetic or thermodynamic restriction to proton flow. The elevated  $\Delta pH$  and consequent low luminal pH can activate energy-dependent quenching mechanisms that dissipate light energy to safeguard the photosystems.

If TPU capacity is inexpensive in terms of nitrogen cost, but is typically just above ambient photosynthetic rates, we would expect that TPU capacity is plastic. It has been found that TPU capacity is flexible, and in many cases changes in response to environmental conditions.

Plants grown at low temperature can develop additional sucrose synthesis enzymes (Cornic & Louason, 1980; Guy et al., 1992; Holaday et al., 1992) which alleviates cold-induced TPU limitation (Sage & Sharkey, 1987). Plants with reduced access to  $CO_2$  have reduced TPU capacity to match their lowered photosynthetic rate (Sharkey & Vasey, 1989; von Caemmerer & Farquhar, 1984). It has, therefore, been shown that TPU capacity can both increase and decrease in response to environmental conditions. This is reflected in environmental surveys, and plants have rarely been found to be TPU limited under ambient conditions in the field (Ellsworth et al., 2015; Sage & Sharkey, 1987). For this reason, TPU limitation is often not included in global models of photosynthesis (Lombardozi et al., 2018; Rogers et al., 2020).

Ideally, if a plant is TPU limited, it will increase its TPU capacity to maximise the overall rate of photosynthesis, but it is also possible that rubisco capacity and electron transport capacity will be decreased to match TPU capacity. In practice the TPU behaviour is induced by reducing the temperature, lowering the oxygen partial pressure, or increasing the partial pressure of  $CO_2$ . Because low temperature has been shown to cause adaptation of TPU capacity, we used high  $CO_2$  to induce TPU limitation to make a comparison of the adaptation. We tested the acclimation of plants to TPU limitation by exposure to elevated  $CO_2$  to determine whether plants eventually stop being TPU limited, and if they achieve this by increasing their TPU capacity. In addition, we established a timeline of the regulatory features surrounding TPU limitation, from how the plant handles the initial influx of energy until the plant engages slower regulatory features, such as rubisco deactivation and energy-dependent quenching.

## 2 | METHODS

### 2.1 | Growth of plant materials

*Nicotiana benthamiana* was found to exhibit very reproducible TPU behaviour and so was the species used here. Seeds were germinated in 2 L pots of potting media consisting of 70% peat moss, 21% perlite, and 9% vermiculite (Suremix; Michigan Grower Products Inc.) in a greenhouse from June-August. This greenhouse is located at 42°43'N, 84°28'W, East Lansing, Michigan, USA. Typical daylight PAR levels inside the greenhouse were between 300 and 700  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and the temperature was controlled to 27°C during the day and allowed to fall to as low as 18°C at night, though nighttime temperatures typically did not reach this low. Plants were watered with half-strength Hoagland's solution (Hoagland & Arnon, 1938) as needed as juveniles and then daily as adults. Plants were used for experiments from 6 to 7 weeks of age.

### 2.2 | Combined gas exchange, fluorescence, and electrochromic shift (ECS) measurements

A LI-COR 6800-12A clear-top chamber (LI-COR Inc.) was modified to incorporate an optical bench for making measurements. The

bottom plate of the clear top chamber was removed and replaced with a 3D-printed backplate with an infrared and an optical detector. These detectors were connected to an Idea Spec (Hall et al., 2013). A front plate was also 3D printed to secure a scattering optic to the top of the 6800-12A. Behind the scattering optic was an array of LEDs containing eight actinic blue and red LEDs, capable of producing up to  $2500 \mu\text{mol m}^{-2} \text{s}^{-1}$  constantly or a saturating flash up to  $15\,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ , at an approximately 90% red/10% blue ratio. Measuring LEDs for ECS were 520 nm, with 505 nm and 535 nm as correction wavelengths for zeaxanthin and  $q_E$  effects on the 520 nm signal. Measuring lights for PSI measurements were at 820 nm with 910 nm as a correction wavelength. Measurements of chlorophyll fluorescence used the 520 nm LEDs as an excitation light. Measurements of PSI were performed according to Kanazawa et al. (2017) and measurements of ECS were performed according to Takizawa et al. (2007). These modifications to the chamber allowed high precision optical measurements simultaneous with high precision gas exchange measurements, especially  $A$  and intercellular  $\text{CO}_2$  partial pressure ( $C_i$ ) allowing construction of  $A/C_i$  curves.

### 2.3 | Protocol for repeated $A/C_i$ measurements

Repeated  $A/C_i$  responses were determined on the same leaves to test the acclimation of the major  $A/C_i$  curve parameters to TPU-limiting conditions. Plants were exposed to the high  $\text{CO}_2$  partial pressure to induce TPU. The  $A/C_i$  measurements were performed by a visual basic script controlling a set of flow controllers attached to the inlet of a LI-COR 6800. Oxygen was held constant at 210 kPa (21%),  $\text{CO}_2$  was varied to achieve ranges of  $\text{CO}_2$  mole fractions from 50 to 1500 ppm, and humidified nitrogen made up the balance (It is generally preferred to express gas levels as partial pressure but since we mixed gases by volume we use mole fractions generally  $\mu\text{mol mol}^{-1}$ , ppm). Plants were acclimated to ambient  $\text{CO}_2$  (about 400 ppm) for an hour after dawn before the first curve. During the first 15 min of this acclimation period, light levels were gradually raised until  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ . After that point,  $A/C_i$  curves were measured every 2.5 h until an hour before dusk, and the plants were given 8 h of darkness, then an hour of acclimation to the light the next day before resuming curves every 2.5 h. From the end of the first curve until the end of the experiment, plants were subjected to an experimental level of  $\text{CO}_2$ , either 150 ppm (low), 400 ppm (ambient), or 1500 ppm  $\text{CO}_2$  (elevated). Curves were analyzed according to Gregory et al. (2021).

### 2.4 | High density optical measurements

To create the timeline of optical measurements after the imposition of TPU limitation, plants were first acclimated at 400 ppm  $\text{CO}_2$  and  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  light in the chamber of the modified 6800-12A

clear-top chamber. A list of times from 10 to 200 s was randomised by script, and for each time interval a second script was run. This script controlled a flow controller to rapidly switch the plant from 400 ppm  $\text{CO}_2$  to 1500 ppm  $\text{CO}_2$ . A measurement of ECS was made by dark interval relaxation kinetics (DIRK) (Takizawa et al., 2007) after the chosen time period. Ten seconds later, a measurement of PSI oxidation state decay and reoxidation by saturation flash was made. Leaves were then incubated at 400 ppm  $\text{CO}_2$  for 10 min. The process was then repeated, but instead of a DIRK to measure ECS, a saturation flash was given to assess PSII characteristics, including the quantum efficiency of photosystem II ( $\phi_{II}$ ) (Baker, 2008) and oxidation status of the quinone  $Q_a$ , measured as  $q_L$  (Kramer et al., 2004). Leaves were again incubated at 400 ppm  $\text{CO}_2$  for 10 min. This process was repeated for every time interval in the list. This protocol was used so that the disruptive saturating flash did not affect subsequent measurements in the time course.

### 2.5 | Rubisco activation state assay

*N. benthamiana* leaves were incubated at 400 ppm  $\text{CO}_2$  until they reached steady state photosynthesis, then the  $\text{CO}_2$  was switched to 1500 ppm for a specified time. The plants were then sampled by freeze-clamp (Schrader et al., 2004). Rubisco activation state was assayed according to Li et al. (2019).

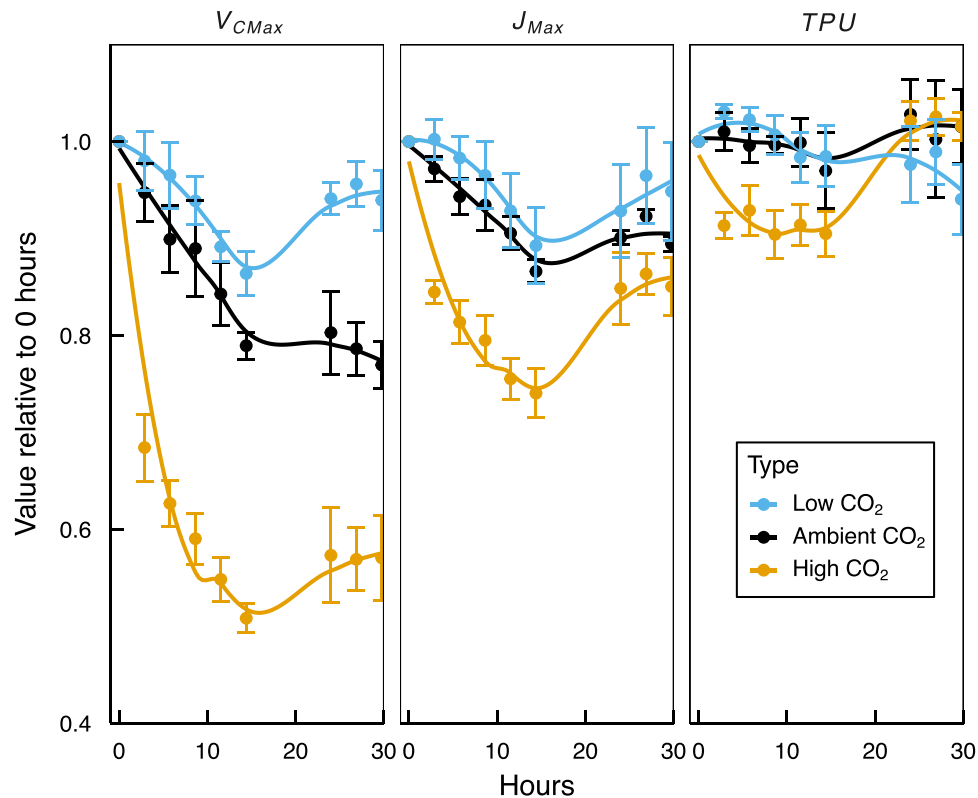
### 2.6 | Statistical treatments

All statistical analysis was done in the R base stats package. Normality was tested using the Shapiro-Wilk's test (Shapiro test in R), and further statistics were only considered where the hypothesis of normality was not rejected ( $p > 0.05$ ). All  $t$  tests ( $t$  test in R) were made without assuming equal variance and instead using the Welch degrees-of-freedom approximation. For  $A/C_i$  fit parameters  $V_{cmax}$ ,  $J$ , and TPU, numbers were normalised to the zero time point. No other data transformation was performed before statistical tests. Scripts are available upon request.

## 3 | RESULTS

### 3.1 | Intermittent $A/C_i$ curves show adaptation of photosynthetic processes over time

Plants were exposed to each  $\text{CO}_2$  condition for 30 h, and  $A/C_i$  responses were determined before the start and then every 2.5 h after imposing the  $\text{CO}_2$  treatment to assess any changes in photosynthetic parameters (Figure 1). After a 16-h day, plants were given an 8-h night and then an hour to acclimate to the light before resuming photosynthetic experiments. For all three conditions,  $V_{cmax}$  and  $J$ , as determined by the fitting routine of Gregory et al. (2021), declined over the first day. The decline in  $V_{cmax}$  and  $J$  was comparable for the low  $\text{CO}_2$  and ambient  $\text{CO}_2$  conditions, and the difference



**FIGURE 1** Plants were exposed to high (1500 ppm) ambient (400 ppm) or low (150 ppm) CO<sub>2</sub> for 30 h, including an 8-h dark period during the typical night hours, with A/C<sub>i</sub> curves performed every 2.5 h. The A/C<sub>i</sub> curves were fit according to Gregory et al. (2021) and the three primary fit parameters,  $V_{cmax}$ ,  $J$ , and TPU are plotted relative to an A/C<sub>i</sub> curve run before treatment began (0 time point). Five separate plants were used for each treatment, and the error bars represent mean and standard error. TPU, triose phosphate utilisation

between the two treatments was not significant at  $p \leq 0.05$  by two-sided  $t$  test at any time in the first day except for in  $V_{cmax}$  at 12.5 h. There was a significant difference ( $p \leq 0.05$  by two-sided  $t$  test) between the decline in  $V_{cmax}$  and  $J_{max}$  in elevated CO<sub>2</sub> condition compared to either of the other treatments at every sampled treatment time during the first day, excluding the 0-time point before treatment began.  $V_{cmax}$  for the elevated CO<sub>2</sub> plants declined by 25% before the first treated A/C<sub>i</sub> and did not recover even overnight.  $J$  for the elevated CO<sub>2</sub> condition did not fully recover overnight, indicative of persistent photoinhibition. TPU capacity decreased relative to the pre-treatment A/C<sub>i</sub> at all timepoints during treatment in the first day for elevated CO<sub>2</sub> treated plants,  $p < 0.05$  by one-sided  $t$  test.

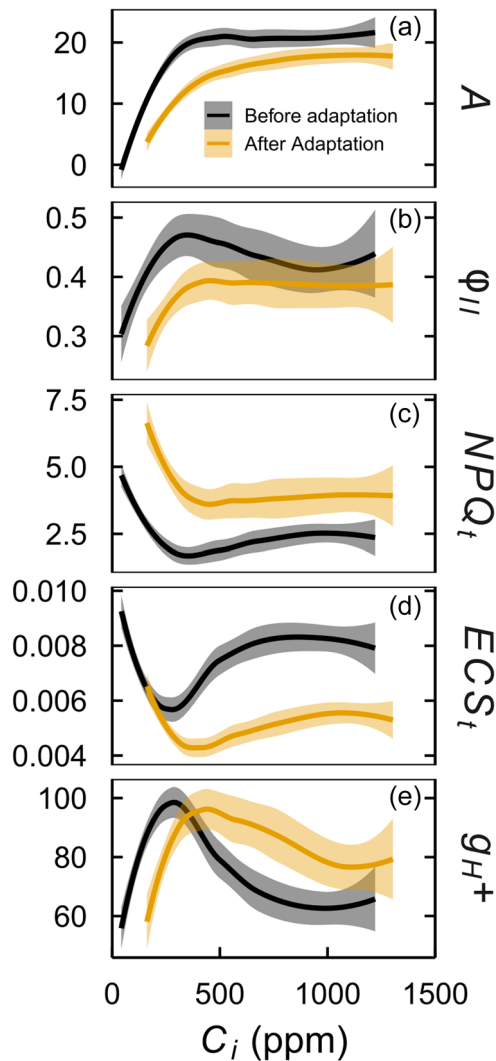
### 3.2 | After acclimation to elevated CO<sub>2</sub>, plants no longer appear to be TPU-limited

After the 30-h acclimation period, plants no longer showed the responses to elevated CO<sub>2</sub> that indicate TPU limitation. The reduced or inverse response of A to CO<sub>2</sub> was gone (Figure 2). The expected CO<sub>2</sub>-dependent decline of  $\phi_{II}$  was absent after acclimation. Elevated nonphotochemical quenching (NPQ<sub>t</sub>) at high CO<sub>2</sub>, one of the effects that causes the decline in  $\phi_{II}$ , was gone after acclimation. TPU

limitation is expected to decrease proton conductivity across the thylakoid membrane ( $g_{H^+}$ ), causing an increase in PMF (measured as total ECS, ECS<sub>t</sub>). These effects were still evident after adaptation, but adapted plants showed a reduced response of ECS<sub>t</sub> to increasing CO<sub>2</sub> relative to the pre-adaptation plants (Figure 2d,e). The increase in ECS<sub>t</sub> is lower at all [CO<sub>2</sub>] greater than 400 ppm for adapted plants. Based on the absence or decline of these physiological effects, we argue that the plants no longer experienced TPU limitation after acclimation, though not as a result of increased TPU capacity.

### 3.3 | Lowered rubisco activation state was a persistent effect in adaptation to TPU stress

Rubisco activation state was measured over the course of adaptation to elevated CO<sub>2</sub>. Rubisco activation state declined over a few min (Figure 3c) and remained low over the course of adaptation (Figure 3a). The prominent decline in  $V_{cmax}$  is also an indicator of reduced rubisco activation state (Figure 1). In addition, the total activatable rubisco activity decreased over the course of adaptation to elevated CO<sub>2</sub> (Figure 3b). The decline in rubisco activation state caused by 2.5 h elevated CO<sub>2</sub> is recoverable within 10 min (Figure 3d).



**FIGURE 2**  $\text{CO}_2$  assimilation and optical measurements from an  $A/C_i$  curve before and after a 30 h 1500 ppm  $\text{CO}_2$  treatment. After 30 h in elevated  $\text{CO}_2$ , parameters show acclimation to TPU-limiting conditions, including reduced response of assimilation (a),  $\phi_{II}$  (b),  $\text{NPQ}_t$  (c), and  $\text{ECS}_t$  (d) to increasing  $\text{CO}_2$ . The clouds are LOESS fitting (Local Estimation of Scatterplot Smoothing) 95% CI  $n = 5$ . CI, confidence interval; TPU, triose phosphate utilisation [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

### 3.4 | Detailed kinetics of photosynthetic processes in response to $\text{CO}_2$ pulses

A step change in  $\text{CO}_2$  to levels that cause TPU limitation induced kinetics in the electron transport chain (Figure 4). There were several kinetic stages. At first, the elevated  $\text{CO}_2$  allowed a faster use of electrons, and PSI became oxidised (Figure 4b). The plant had not yet entered TPU limitation, as indicated by the high proton conductivity of the ATP synthase ( $g_{H^+}$ ) (Figure 4e). The second phase (Figure 4, blue), beginning 40 s after the step change in  $\text{CO}_2$  flow and persisting until 80 s after the beginning of  $\text{CO}_2$  flow, was characterised by the reduction of  $Q_a$  (Figure 4c) [ $q_L$

is a fluorescence-based measure that increases with increased oxidation of  $Q_a$  (Kramer et al., 2004)]. The reduction of  $Q_a$  caused an increase in  $\phi_{\text{NPQ}}$  (Figure 4g) and a decrease in  $\phi_{II}$  (Figure 4f) even though  $\text{NPQ}_t$  (Figure 4i) [measured using the  $\text{NPQ}_t$  parameter (Tietz et al., 2017)] did not respond within this timeframe.

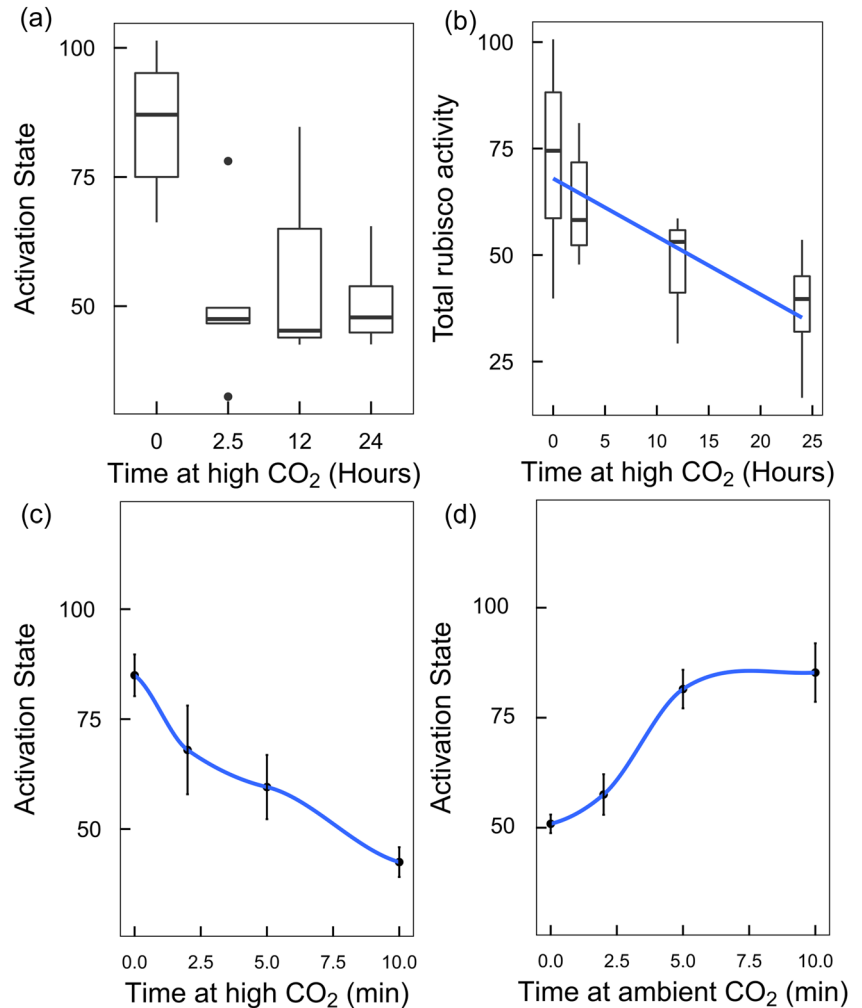
The reduction of  $Q_a$  was correlated with the reduction of PSI. The kinetic constant for reduction of PSI by cytochrome  $b_6/f$  ( $k_{et}$ , Figure 4d), decreased, so we conclude that the reduction of PSI was not due to excess electrons being transported downstream. Therefore, the reduction of PSI must be due to an acceptor-side limitation of PSI, indicating a lack of availability of  $\text{NADP}^+$ . In the same stage, a decline in  $g_{H^+}$  can be seen, decreasing by over 50% (Figure 4e). The low  $g_{H^+}$  that was observed has been shown to be associated with TPU limitation (Kiirats et al., 2009; Yang et al., 2016). The third kinetic stage (Figure 4, green) began 80 s after the beginning of the  $\text{CO}_2$  step change and exhibited slower regulatory mechanisms. PMF (Figure 4h) increased up to this point, and continued to increase during this phase, which caused an increase in energy-dependent  $\text{NPQ}_t$  (Figure 4i), and a decrease in  $k_{et}$  (Figure 4d). These mechanisms prevent electrons from reaching PSI, alleviating the over-reduction of PSI. After the PMF increased sufficiently, photosynthesis entered a new steady-state (Figure 4, red).

The interpretation of PSI acceptor-side limitations is supported by the observed response of PSI oxidation state to flashes of saturating light (Figure 5). Leaves were given a brief dark interval to allow reduction of PSI and then PSI was oxidised by a saturating flash. When tested in the middle of TPU-induced transients (Figure 5a), PSI did not remain oxidised by the saturating flash, and instead began re-reducing due to inability to pass electrons to  $\text{NADP}^+$ . Tests made some time after the onset of TPU-limiting conditions showed less re-reduction (Figure 5b), and with more time, re-reduction was much less prominent (Figure 5c).

### 3.5 | Transient response to TPU limitation is lost after acclimation

Five plants were tested for transient responses to TPU-limiting conditions before and after a 24-h acclimation to elevated  $\text{CO}_2$  (Figure 6). A list of timepoints from 10 to 200 s was randomised by R script; for each plant the randomisation was different. For each timepoint, plants were given 10 min at ambient  $\text{CO}_2$  (400 ppm) before pulsing with high  $\text{CO}_2$  (1500 ppm) at the end of which chlorophyll fluorescence parameters were measured. Non-adapted plants exhibited a transient reduction of  $Q_a$  to a minimum of 21% following the introduction of TPU-limiting conditions, resulting in partitioning of energy into  $\text{NPQ}$  rather than photochemistry. After adaptation, plants did not exhibit reduction of  $Q_a$  significantly below the steady-state value in the elevated  $\text{CO}_2$  environment.

**FIGURE 3** TPU limitation causes reduced rubisco activation state percentage that persists for an extended period. Rubisco activation state (a) and total activity (b) are measured at 0, 2.5, 12, and 24 h to show changes in activity over the course of a day's acclimation. Slope of the decline in total rubisco activity is significant at  $p < 0.05$ . Rubisco activation state decreases to its minimum within 10 min (c), and activation state is not significantly different after 10 min and 2.5 h (d, 0 time). After 2.5 h at elevated  $\text{CO}_2$ , activation state recovers completely after 5 min (d), with activation state 5 min into recovery not significantly different from the 0 min unadapted activation state (c, 0 time) by two-sided  $t$  test. TPU, triose phosphate utilisation [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



## 4 | DISCUSSION

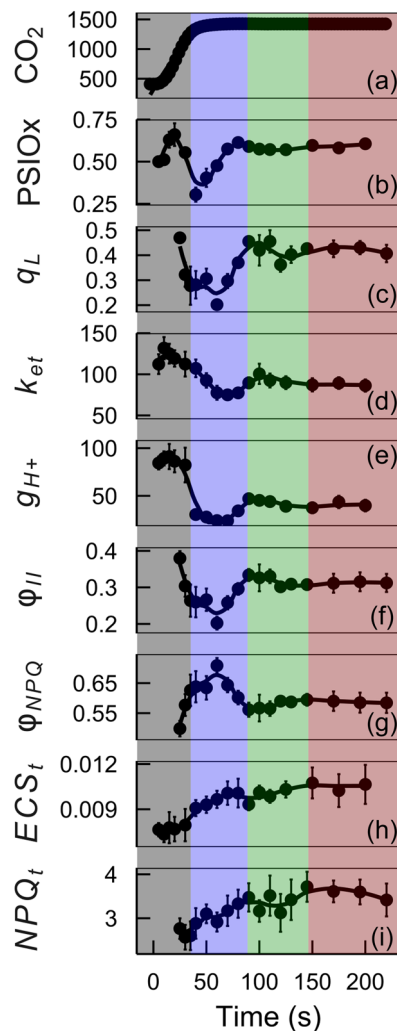
### 4.1 | Fast onset kinetics in responses to TPU limitation are directed by electron build-up on $Q_a$

When plants were subjected to TPU-limiting conditions, the most immediate effects were transient changes in the redox states of electron transport components. It is known that while TPU-limited, increasing  $\text{CO}_2$  levels cause a reduction in  $\phi_{II}$  because, while  $A$  cannot increase, the rate of photorespiration will go down (Sharkey et al., 1988; Stitt & Grosse, 1988; Stitt, 1986). This, combined with the common observations of elevated  $PMF$  and non-photochemical quenching during TPU limitation, indicates the importance of  $q_E$  in dissipating absorbed light energy when electron transport capacity exceeds TPU capacity. However,  $q_E$  does not activate instantaneously, with the xanthophyll cycle and PSBS recruitment to the reaction centre operating on the minutes timescale (Li et al., 2002). Therefore, we could reasonably predict excess accumulation of electrons on electron transport intermediates and PSI electron acceptors. Reduction of  $Q_a$  decreases the quantum efficiency of photochemistry because PSII cannot accept any more energy. The energy that would be going towards

photochemistry is instead shunted to nonphotochemical quenching, resulting in an increased yield of nonphotochemical quenching. This means that  $\phi_{NPQ}$  increases even though  $NPQ_t$  changes on a slower timescale. Immediately after entering TPU limitation, electrons build up on the electron transport chain due to decreased electron sink strength, and the bulk of the excess energy is most immediately handled by controls within the electron transport chain.

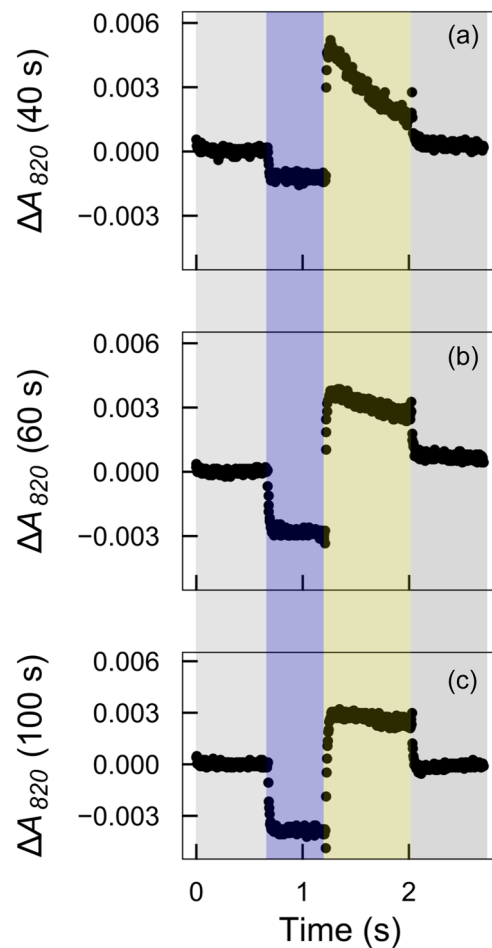
Though the reduction of  $Q_a$  reduces the yield of photochemistry, the reduction of PSI following the imposition of TPU limitation is more concerning. Acceptor-side limitation of PSI is highly stressful due to the accumulation of ROS (Li et al., 2009) and the inability of PSI to repair itself (Sonoike, 1996, 2011). Electron transfer to PSI from the cytochrome  $b6f$  complex is slowed by elevated  $PMF$  due to the requirement to oxidise plastoquinol (Kramer & Crofts, 1993; 1996). We found, however, that  $PMF$  does not build up fast enough to adjust to the limiting demand from the Calvin-Benson cycle and regulate electron flow to PSI, and electrons do indeed accumulate on PSI. This is not due to an accelerated rate of PSI reduction through the cytochrome  $b6f$  complex ( $k_{et}$ , Figure 4), so it must instead be due to an acceptor side limitation of PSI because of a lack of  $\text{NADP}^+$ . Increasing  $[\text{CO}_2]$  under TPU limitation reduces the





**FIGURE 4** Plants are given a step change in  $[CO_2]$  from 400 to 1500 ppm, which induces oscillations in electron transport (a). Plants are held at 1500 ppm  $CO_2$  for a randomised length of time (x-axis) then measurements of their PSI and PSII activity are taken, along with electrochromic shift. The data is divided into four putative kinetic periods. In the first phase (grey region), photosynthesis is unlimited by TPU, and PSI becomes more oxidised (b). The second phase (blue) is the onset of TPU limitation and notably affects proton flow across the thylakoid membrane ( $g_{H^+}$ , e), PSI oxidation state (b) and  $Q_a$  oxidation state (measured as  $q_L$ , c). Reduction of  $Q_a$  causes energy diversion from photochemistry ( $\phi_{II}$ , f) to nonphotochemical quenching ( $\phi_{NPQ}$ , g). The third phase (green) begins when proton-motive-force (measured as  $ECS_t$ , h) increases along with energy dependent quenching ( $NPQ_t$ , i) and photoprotection at cytochrome  $b6f$  complex (d). Finally, electron transport enters a new steady-state (red). Dots represent mean value and error bars are standard error,  $n = 5$ . TPU, triose phosphate utilisation [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

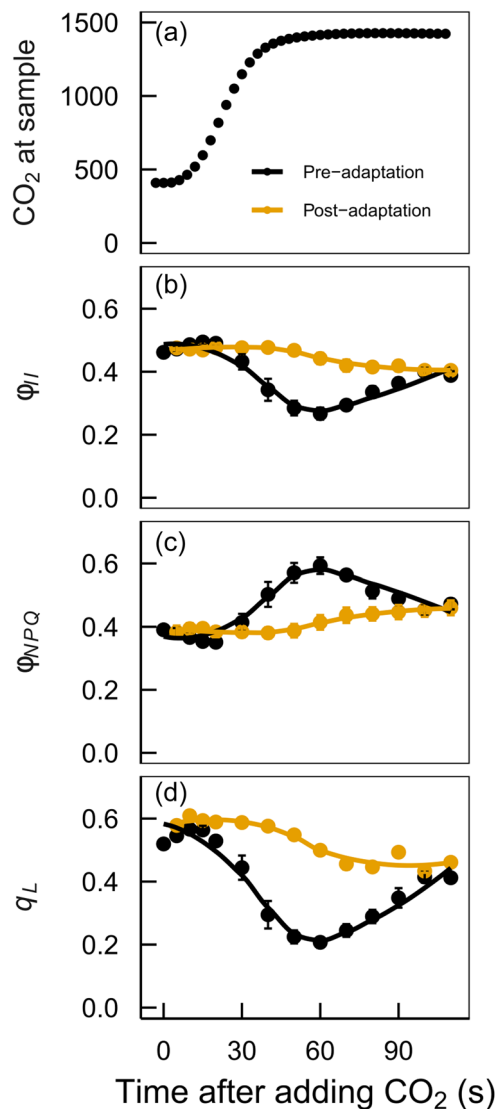
rate of photorespiration, and if A cannot increase due to TPU limitation the overall rate of consumption of both ATP and NADPH decreases. The NADPH pool turnover (half-time  $0.01 s^{-1}$ ) is faster than that of ATP (half-time  $0.28 s^{-1}$ , Arrivault et al., 2009), so the reduced consumption of electron transport products will affect



**FIGURE 5** Three example traces of PSI measurements from oscillations in PSI reduction induced by step change in  $CO_2$  from 400 to 1500 ppm, which demonstrate varying levels of re-reduction during saturating flashes. Plants under actinic light (grey) were subjected to a 0.5 s dark period (blue), causing reduction of PSI ( $\Delta A_{820}$  decreases). Then, a saturating flash is applied (yellow) to oxidise PSI ( $\Delta A_{820}$  increases), before returning to steady state. A saturating flash should fully oxidise PSI, but kinetics in electron transport can change this. (a) Extreme re-reduction of PSI can be seen during a saturation flash when PSI is most reduced, 40 s after beginning an elevated  $CO_2$  pulse. (b) Less re-reduction of PSI during a saturation flash is seen when PSI is less reduced, 60 s after a  $CO_2$  step change. (c) 100 s after the  $CO_2$  step change, PSI re-reduction is much reduced [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

NADP<sup>+</sup> availability first. Restriction of NADPH oxidation has been suggested previously as the cause of oscillations in TPU limitation (Furbank et al., 1987). The restriction of NADP<sup>+</sup> flux can be seen in the re-reduction of PSI during a saturation flash at the point of greatest PSI reduction (Figure 5). During this saturation flash, light is in excess of what is required to oxidise PSI, and the only limitation would be the electron carriers removing the electrons from PSI.

The accumulation of electrons on electron carriers of the electron transport chain is resolved by slower regulation. PMF increases, causing a decrease in  $k_{et}$  and an increase in  $NPQ_t$ . As these slower control mechanisms take hold, the transients in the other



**FIGURE 6** Oscillations are not seen following a step change in  $\text{CO}_2$  in plants that have acclimated to elevated  $\text{CO}_2$  for 30 h. The hallmark reduction of  $Q_a$ , measured here as  $q_L$ , is not seen, and so more energy is not diverted into non-photochemical quenching ( $\Phi_{NPQ}$ ). Dots and bars represent mean  $\pm$  standard error,  $n = 5$  [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

parameters slow and then stop. This is one example of damped oscillations, commonly found associated with TPU limitation (Ogawa, 1982; Sivak & Walker, 1986, 1987). The oscillations are caused by perturbations in the electron requirements of the Calvin-Benson cycle forcing  $Q_a^-$  based control of electron transport; they are damped by the onset of PMF-based controls of electron transport. Some, but not all measurements of oscillations are consistent with the period and convergence rates in our measurements of oscillations. We therefore propose that electron carrier reduction as described here is responsible for some, but not all, observations of oscillations in TPU limitation.

## 4.2 | Slow-onset regulatory processes control TPU limitation after a period of acclimation

On the minutes timescale, TPU-limited photosynthesis is regulated by rubisco deactivation, photosynthetic control at the cytochrome  $b_6f$  complex, and  $q_E$ . Rubisco deactivation begins within minutes and persists for at least a day (Figure 3). It is known that photosynthetic control and  $q_E$  are induced by acidification of the thylakoid lumen. The mechanism of rubisco deactivation is less clear. Study has been made on the deactivation of rubisco under elevated temperature (Salvucci & Crafts-Brandner, 2004) but no clear mechanistic understanding of deactivation under elevated  $\text{CO}_2$  has been elucidated. Under TPU-limiting conditions, ATP synthase is constricted (Kanazawa & Kramer, 2002; Kiirats et al., 2009; Takizawa et al., 2008) probably due to low phosphate concentration, which leads to a lower ATP/ADP ratio (Sharkey et al., 1986b; Furbank et al., 1987; Stitt, 1986) and, therefore, reduced rubisco activase activity. We measured a reduction in total rubisco activity after activation with 6-phosphogluconate (Figure 3b), which could be caused by tight binding inhibitors (Keys et al., 1995; Parry et al., 1997; Paul et al., 1996). This can contribute to reduced rubisco activity. Reversible deactivation of rubisco is the primary contributor to the reduction in  $V_{cmax}$  measured over the course of acclimation (Figure 1).

Over time, photoinhibition becomes responsible for dissipating more excess energy, supplanting  $q_E$ . Measured  $J$  at  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  began decreasing quickly and did not recover fully overnight (Figure 1). In addition, after acclimation, total  $NPQ_t$  was higher at all levels of  $\text{CO}_2$ , and  $NPQ_t$  did not increase at elevated  $\text{CO}_2$ . PMF ( $ECSt$ ) is overall lower and has a reduced response to increasing  $\text{CO}_2$ . This indicates that  $q_E$  is becoming less important in energy flux compared to  $q_i$ , especially in response to TPU limitation. The NPQ must come from other sources, such as quenching from photoinhibition or state transitions. State transitions are somewhat limited in higher plants, with only 15%–20% of the light harvesting complex capable of relocation (Rochaix, 2011), so photoinhibition is the most likely cause. The energy dissipation due to photoinhibition is enough to protect the photosystems, which makes  $q_E$  unnecessary.

Acclimation to TPU limitation requires balancing of both carbon and energy flux. At the end of acclimation, we found that energy flux is balanced by photoinhibition, and that carbon flux is balanced by rubisco deactivation. These two systems work synergistically. Rubisco deactivation reduces the potential demand for ATP and NADPH when  $\text{CO}_2$  fixation could exceed the potential for end-product production. Control of electron transport by photoinhibition decreases the potential to overload the electron transport chain from the beginning. In this way, even though photoinhibition is rightly considered a negative effect on the plant, it is effective in protecting PSI; PSII is damaged, but there are effective repair mechanisms for PSII (Ohad et al., 1984; Sonoike, 1996; Vass et al., 1992). These two effects combine to reduce pressure on inorganic phosphate pools by reducing the potential use of phosphate from both sides.



### 4.3 | After a long enough period of adaptation, plants no longer appear to be TPU-limited

TPU limitation is characterised by the responses of photosynthesis to increasing CO<sub>2</sub> (McClain & Sharkey, 2019). Once the plant becomes TPU-limited, elevating CO<sub>2</sub> results in elevated PMF and NPQ, while reducing  $\phi_{II}$  and  $g_{H^+}$  through the thylakoid membrane. In addition, the shape of the  $A/C_i$  curve is distinct: with increasing CO<sub>2</sub>,  $A$  remains constant or marginally decreases due to reduced export of photorespiratory intermediates (Busch et al., 2018). After 30 h of acclimation to elevated CO<sub>2</sub>, evidence of TPU is gone (Figure 2). NPQ is overall higher but does not show the characteristic response to increasing CO<sub>2</sub> typical of TPU limitation.  $\phi_{II}$  is lower at some CO<sub>2</sub> levels and not significantly different at others, but the characteristic shape of the curve is lost after acclimation. Because TPU limitation is characterised by these responses, we argue that the plants do not become TPU limited by elevated CO<sub>2</sub> after acclimation. TPU limitation happens in three phases: first, an acute condition, where phosphate incorporation and release are most imbalanced, resulting in dynamic fluctuations in electron carrier redox state and ATP availability. Second, a position of regulatory control, where rubisco deactivation and energy-dependent quenching dominate the observable phenomena associated with TPU limitation. Third, the plant will adapt to the conditions it is embroiled in, and the middle timescale regulation is phased out by greater adaptive responses that prevent TPU limitation from occurring.

It is generally thought that extended periods of time in high light and low CO<sub>2</sub> will cause damage to the photosynthetic apparatus, but data reported here show that extended periods of high CO<sub>2</sub> are deleterious while low CO<sub>2</sub> are not as bad. This is interpreted as TPU being a stressful condition that causes regulatory responses that result in a loss of TPU behaviour. The acclimation shown here prevents plants from experiencing TPU stress.

Debate has recently surfaced about the relevancy of TPU limitation to global models (Lombardozi et al., 2018; Rogers et al., 2020). TPU limitation is rarely diagnosed as the limiting factor of steady-state photosynthesis in the wild (Sage & Sharkey, 1987). We believe that this is due to the relatively fast adaptation to TPU limiting conditions. Within a day of acclimation to very high CO<sub>2</sub>, TPU limitation would not be diagnosable from gas exchange or fluorescence analysis. TPU limitation would only happen transiently. For this reason, we agree that TPU limitation as an explicit parameter of photosynthesis need not factor into global models of photosynthesis. However, it is important as a component of the regulatory network of photosynthesis.

It is currently unclear as to why TPU capacity did not increase in response to elevated CO<sub>2</sub> (Figure 1). If maximising photosynthesis were the only concern, the plant would produce extra enzymes for processing end products to relieve TPU limitation instead of reducing other photosynthetic capacities. Some experiments have been done previously connecting TPU capacity with low temperature, another primary cause of TPU limitation (Sharkey & Bernacchi, 2012) due mostly to the high temperature sensitivity of sucrose-phosphate

synthase (Stitt & Grosse, 1988). Plants grown in low temperature produced significantly more sucrose synthesis enzymes (Guy et al., 1992; Holaday et al., 1992; Hurry et al., 2000). We know, therefore, that plants which have been TPU limited can produce more end-product-synthesis enzymes, so it seems like an obvious inefficiency for plants to lose photosynthetic capabilities. This conundrum may reflect the interaction between plant growth and photosynthesis. Some analyses indicated that photosynthetic rate is not the best predictor of plant growth (Körner, 2015). Factors controlling growth rate and photosynthetic rate may not always work in concert. Growth is more temperature sensitive than is photosynthesis and so it may be that at low temperature growth limits photosynthesis while at high temperature photosynthesis limits growth. In this case, while the plant may look like it is performing inefficiently, it may simply be growing as fast as possible, and any additional photosynthesis would not be useful. Thus far it has been difficult to establish explicit causality connecting sink regulation to TPU limitation (Paul & Foyer, 2001) but efforts have been reported (Dingkuhn et al., 2020; Fabre et al., 2019). Recent work on SnRK1, the Target of Rapamycin complex, and interactions with trehalose 6-phosphate signalling may eventually help explain the interaction between plant growth and photosynthetic rate (Brunkard, 2020; Lastdrager et al., 2014; Peixoto et al., 2021; Shi et al., 2018; Smeekens et al., 2010; Sulpice et al., 2009).

## 5 | CONCLUSIONS

Photosynthesis is highly adaptive to the environment, and in TPU-limiting conditions experiences a series of regulatory steps to alleviate the stress along the electron transport chain. These steps can be organised into a timeline. At first, electrons build up along the electron transport chain, and reduction of Q<sub>a</sub> causes extra energy to be funneled into nonphotochemical quenching. This causes transients in photosynthesis, which are damped after a few minutes by accumulation of PMF, causing elevated energy-dependent quenching and photoprotection at the cytochrome *b<sub>6</sub>f* complex, accompanied by reduction in rubisco activation state. Over a longer period of time, energy-dependent quenching decreases and is supplanted by photoinhibition. The accumulation of these regulatory mechanisms causes the plant to no longer be TPU limited. Counterintuitively, the plant did not increase its TPU capacity, but instead limited the photosynthetic rate by rubisco deactivation and electron transport regulation.

The disappearance of TPU limitation over 30 h of adaptation justifies the removal of TPU limitation from global models. Plants that are TPU-limited will eventually not be TPU limited, through a combination of regulatory means. However, TPU limitation is still an important part of photosynthetic regulation and cannot be disregarded in experimental design or data analysis. The occurrence of TPU limitation in the field is probably very low due to the swift adaptation demonstrated here, but in artificial experiments is easy to provoke. In FACE experiments (Allen et al., 2020), or experiments

that involve low temperature many of the effects studied may be caused by TPU limitation or the acclimation to TPU limitation. In other cases, sugar signalling may match photosynthesis to growth without explicit TPU limitations.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

All data are available upon request to the corresponding author. All data are available from Dryad <https://doi.org/10.5061/dryad.0p2ngf24r>.

## ORCID

Alan M. McClain  <http://orcid.org/0000-0002-4989-1436>

David M. Kramer  <http://orcid.org/0000-0003-2181-6888>

Thomas D. Sharkey  <http://orcid.org/0000-0002-4423-3223>

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#### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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