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

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Abstract

Triose-phosphate utilization (TPU) limits the maximum rate at which plants can photosynthesize. 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 $_2$ concentrations. Plants held at 1500 ppm CO $_2$ were initially TPU limited. After 30 hours 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 $_2$ first caused PSI to be oxidized 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.

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Triose-phosphate utilization (TPU) limits the maximum rate at which plants can photosynthesize. 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

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KEY WORDS

Thylakoid ATP synthase, Energy-dependent exciton quenching, Photosynthetic control, Rubisco activation, triose phosphate use (TPU)

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 modeled assuming ribulose 1,5-bisphosphate (RuBP)-saturated rubisco kinetics; the RuBP regeneration limitation, where carbon dioxide uptake is modeled assuming a fixed rate of RuBP use as allowed by the production of electron transport products, ATP and NADPH; and the triose phosphate utilization (TPU) limitation, where carbon dioxide uptake is modeled as the rate of production of end products, freeing inorganic phosphate from organic phosphates (McClain & Sharkey 2019). The TPU limitation is unique among the three biochemical limitations in that it is limited by downstream processes, rather than just at rubisco. The dislocation of the limitation from rubisco means that 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 ($q_{\boldsymbol{E}}$) is activated (Sharkey, Berry & Sage 1988) by elevated ΔpH across the thylakoid membrane, one component of proton-motive force (PMF) (Kramer & Crofts 1996). The elevated ΔpH 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, Seemann & Berry 1986a; Socias, Medrano & Sharkey 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 (Powles 1984; Pammenter, Loreto & Sharkey 1993; Li, Müller-Moulé, Gilmore & Niyogi 2002).

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, Laisk, Oja & Pärnik 1977) or CO_2 (Jolliffe & Tregunna 1973), it is easier to assess by the decline in electron transport rate associated with $q_{\boldsymbol{E}}$ when CO_2 is increased or O_2 is decreased. The appearance of transient effects on photosynthesis associated with TPU limitation (Ogawa 1982; Walker, Sivak, Prinsley & Cheesbrough 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.

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 andq_E is increased reducing the efficiency of nitrogen use in both carbon metabolism and electron transport. Because TPU capacity is relatively cheap and 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, Preiser, Li, Weise & Sharkey 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 (Preiss

1982; Escobar-Gutiérrez & Gaudillère 1997). 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 ΔpH 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, Huber & Huber 1992; Holaday, Martindale, Alred, Brooks & Leegood 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 (von Caemmerer & Farquhar 1984; Sharkey & Vassey 1989). 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 (Sage & Sharkey 1987; Ellsworth, Crous, Lambers & Cooke 2015). For this reason, TPU limitation is often not included in global models of photosynthesis (Lombardozzi *et al.* 2018; Rogers *et al.* 2020).

Ideally, if a plant is TPU limited, it will increase its TPU capacity to maximize the overall rate of photosynthesis, but it is also possible that rubisco capacity and electron transport capacity will be decreased to match TPU capacity. 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.

METHODS

Growth of plant materials

Nicotiana benthamiana was germinated from seed in 2 l pots of potting media consisting of 70% peat moss, 21% perlite, and 9% vermiculite (Suremix; Michigan Grower Products Inc., Galesburg, MI, USA) in a greenhouse from June-August. This greenhouse is located at 42°43'N, 84deg28'W, East Lansing, Michigan, USA. Typical daylight PAR levels inside the greenhouse were between 300-700 μmol $m^{-2}s^{-1}$, 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 6-7 weeks of age.

Combined gas exchange, fluorescence, and electrochromic shift measurements

A LI-COR 6800-12A clear-top chamber (LI-COR Inc., Lincoln, NE, USA) 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 2,500 μmol m^{-2} s^{-1} constantly or a saturating flash up to 15,000 μmol $m^{-2}s^{-1}$, at an approximately 90% red/10% blue ratio. Measuring LEDs for electrochromic shift (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 CO_2 partial pressure (C_i) allowing construction of A / C_i curves.

Protocol for repeated *A/Ci* measurements

Repeated A /C_i responses were determined to test the acclimation of the major A /$C_i$curve parameters to TPU-limiting conditions. 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%), CO_2 was varied to achieve ranges of CO_2mole 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 μmol mol^{-1}, ppm.) Plants were acclimated to ambient 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 μmol m^{-2} 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 CO_2, either 150 ppm (low), 400 ppm (ambient), or 1500 ppm CO_2 (elevated). Curves were analyzed according to Gregory *et al.* (2021).

High density optical measurements

To create the timeline of optical measurements after the imposition of TPU limitation, plants were first acclimated at 400 ppm CO_2 and 1000 μmol m^{-2} s^{-1} light in the chamber of the modified 6800-12A clear-top chamber. A list of times from 10-200 s was randomized 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 CO_2 to 1500 ppm CO_2. A measurement of electrochromic shift was made by dark interval relaxation kinetics (DIRK) (Takizawa *et al.* 2007) after the chosen time period. Ten s later, a measurement of PSI oxidation state decay and reoxidation by saturation flash was made. Leaves were then incubated at 400 ppm 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 (φ_{II}) (Baker 2008) and oxidation status of the quinone Q_a, measured asq_L (Kramer, Johnson, Kiirats & Edwards 2004). Leaves were again incubated at 400 ppm 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 timecourse.

Rubisco activation state assay

N. benthamiana leaves were incubated at 400 ppm CO_2 until they reached steady state photosynthesis, then the CO_2 was switched to 1500 ppm for a specified time. The plants were then sampled by freeze-clamp (Schrader, Wise, Wacholtz, Ort & Sharkey 2004). Rubisco activation state was assayed according to Li *et al.* (2019).

RESULTS

Intermittent *A/Ci* curves show adaptation of photosynthetic processes over time

Plants were exposed to each CO_2 condition for an extended period, and A /C_i responses were determined every 2.5 h to assess any changes in photosynthetic parameters (Fig. 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 CO_2 and ambient CO_2conditions but there was a much greater decline in the elevated CO_2 condition. V_{cmax} for the elevated CO_2 plants declined by 25% before the first treated A /C_i and did not recover even overnight. J for the elevated CO_2 condition recovered somewhat overnight but not entirely, indicative of some persistent photoinhibition. The TPU capacity of the elevated CO_2 plants appeared to decline for the first day.

After acclimation to elevated CO_2, plants no longer appear to be TPU-limited

After the 30-h acclimation period, plants no longer showed the responses to elevated CO_2 that indicate TPU limitation. The reduced or inverse response of A to CO_2 was gone (Fig 2a). The expected CO_2-dependent

decline ofφ_{II} was absent after acclimation (Fig 2b). Elevated nonphotochemical quenching (NPQ_t) at high CO_2, one of the effects that causes the decline inφ_{II} , was gone after acclimation (Fig 2c). TPU limitation is expected to decrease proton conductivity across the thylakoid membrane, causing an increase in PMF (measured as total electrochromic shift, ECS_t). These effects were not completely missing but they were decreased (Fig 2d,e). 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.

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_2. Rubisco activation state declined over a few min (Fig 3c) and remained low over the course of adaptation (Fig 3a). The prominent decline in V_{cmax} is also an indicator of reduced rubisco activation state (Fig 1). In addition, the total activatable rubisco activity decreased over the course of adaptation to elevated CO_2 (Fig 3b).

Detailed kinetics of photosynthetic processes in response to CO_2 pulses

A step change in CO_2 to levels that cause TPU limitation induced kinetics in the electron transport chain (Fig 4). There were several kinetic stages. At first, the elevated CO_2allowed a faster use of electrons, and PSI became oxidized. The plant had not yet entered TPU limitation, as indicated by the high proton conductivity of the ATP synthase (g_{H+}). The second phase (Fig. 4, blue), beginning 40 s after the step change in CO_2 flow and persisting until 80 s after the beginning of CO_2 flow, was characterized by the reduction of Q_a [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 φ_{NO} and a decrease inφ_{II} even though NPQ [measured using the NPQ_t parameter (Tietz, Hall, Cruz & Kramer 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_6f(k_{et}), 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. In the same stage, a decline ing_{H+} can be seen, decreasing by over 50%. The lowg_{H+} that was observed has been shown to be associated with TPU limitation (Kiirats, Cruz, Edwards & Kramer 2009; Yang *et al.* 2016). The third kinetic stage (Fig 4, green) began 80 s after the beginning of the CO_2 step change and exhibited slower regulatory mechanisms. Proton-motive force increased up to this point, and continued to increase during this phase, which caused an increase in energy-dependent NPQ_t , and a decrease in k_{et} . These mechanisms prevent electrons from reaching PSI, alleviating the over-reduction of PSI. After the PMF increased sufficiently, photosynthesis entered a new steady-state (Fig. 4, red).

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

Transient response to TPU limitation is lost after acclimation

Plants were tested for transient responses to TPU-limiting conditions before and after a 24-h acclimation to elevated CO_2 (Fig 6). For each time-point, plants were given 10 min at ambient CO_2 (400 ppm) before pulsing with high CO_2 (1500 ppm) at the end of which chlorophyll fluorescence parameters were measured. Time points are scrambled for each plant. 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 intoNPQ rather than photochemistry. After adaptation, plants did not exhibit reduction of Q_a significantly below the steady-state value in the elevated CO_2 environment.

DISCUSSION

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 CO_2 levels cause a reduction inφ_{II} because, while A cannot increase, the rate of photorespiration will go down (Stitt 1986; Sharkey *et al.*1988; Stitt & Grosse 1988). 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 center 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 thatφ_{NPIX} increases even thoughNPQ_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, Wakao, Fischer & Niyogi 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 oxidize 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} , Fig. 4), so it must instead be due to an acceptor side limitation of PSI. Increasing [CO_2] under TPU limitation reduces the 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 $NADP^+$ availability first. Restriction of NADPH oxidation has been suggested previously as the cause of oscillations in TPU limitation (Furbank, Foyer & Walker 1987). The restriction of $NADP^+$ flux can be seen in the re-reduction of PSI during a saturation flash at the point of greatest PSI reduction (Fig 5a). During this saturation flash, light is in excess of what is required to oxidize 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 inNPQ_t . As these slower control mechanisms take hold, the transients in the other 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.

It has been repeatedly observed that TPU limitation is rare to nonexistent in wild plants (Rogers *et al.* 2020), but can often be found in experiments using high levels of light with elevated CO_2, decreased O_2, and/or low temperature. The most stressful moments will be when the plant enters TPU limitation and electrons accumulate on PSI electron acceptors, and therefore would be most stressful when the experimental design would cause fluctuations in photosynthetic abilities. For example, when a plant is held at low temperature, fluctuating light levels would exacerbate the stressful TPU conditions. We believe this can be stressful for plants in FACE experiments, where absolute consistency in CO_2 levels across the field and perfect mixing cannot be reasonably expected. Allen *et al.* (2020) discussed the difficulties in maintaining perfect mixing across a FACE field, and we believe that the stress of entering and exiting TPU due to fluctuating CO_2

levels can be a drag on plant performance, potentially reducing the expected stimulation of growth in high CO_2 FACE experiments (Long, Ainsworth, Leakey, Ort & No 2006).

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 (Fig. 3). Unlike photosynthetic control and q_E , which are induced by acidification of the thylakoid lumen, the mechanism of rubisco deactivation is unknown. Under TPU-limiting conditions, ATP synthase is constricted (Kanazawa & Kramer 2002; Takizawa, Kanazawa & Kramer 2008; Kiirats *et al.* 2009) probably due to low phosphate concentration, which leads to a lower ATP/ADP ratio (Sharkey *et al.* 1986b; Stitt 1986; Furbank *et al.* 1987) and therefore reduced rubisco activase activity. We measured a reduction in total rubisco activity after activation with 6-phosphogluconate (Fig. 3b), which could be caused by tight binding inhibitors (Keys, Major & Parry 1995; Paul *et al.* 1996; Parry *et al.* 1997). 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 (Fig 1).

Over time, photoinhibition becomes responsible for dissipating more excess energy, supplanting q_E . Measured J at 1000 μmol m^{-2} s^{-1} began decreasing quickly and did not recover fully overnight (Fig. 1). In addition, after acclimation, total NPQ_t was higher at all levels of CO_2, and NPQ_t did not increase at elevated CO_2. PMF (ECS_t) is overall lower and has a reduced response to increasing 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 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, Kyle & Arntzen 1984; Vass *et al.* 1992; Sonoike 1996). These two effects combine to reduce pressure on inorganic phosphate pools by reducing the potential use of phosphate from both sides.

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

TPU limitation is characterized by the responses of photosynthesis to increasing CO_2 (McClain & Sharkey 2019). Once the plant becomes TPU-limited, elevating CO_2 results in elevated PMF and NPQ, while reducing φ_{II} and g_{H+} through the thylakoid membrane. In addition, the shape of the A/C_i curve is distinct: with increasing CO_2, A remains constant or marginally decreases due to reduced export of photorespiratory intermediates (Busch, Sage & Farquhar 2018). After 30 h of acclimation to elevated CO_2, evidence of TPU is gone (Fig. 2). Thus, acute TPU limitation is probably a brief condition during which the consumption and production of free phosphate come back into balance, and TPU limitation is instead diagnosed by the regulatory effects that result. Once q_E is supplanted by photoinhibition it becomes difficult to assess TPU limitation.

It is generally thought that extended periods of time in high light and low CO_2 will cause damage to the photosynthetic apparatus, but data reported here show that extended periods of high CO_2 are deleterious while low CO_2 are not as bad. This is interpreted as TPU being a stressful condition that causes regulatory responses that result in a loss of TPU behavior. The acclimation shown here prevents plants from experiencing TPU stress.

Debate has recently surfaced about the relevancy of TPU limitation to global models (Lombardozzi *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_2, 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_2 (Fig. 1). If maximizing 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, Strand, Furbank & Stitt 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 (Fabre *et al.* 2019; Dingkuhn *et al.* 2020). Recent work on SnRK1, the Target of Rapamycin complex, and interactions with trehalose 6-phosphate signaling may eventually help explain the interaction between plant growth and photosynthetic rate (Sulpice*et al.* 2009; Smeekens, Ma, Hanson & Rolland 2010; Lastdrager, Hanson & Smeekens 2014; Shi, Wu & Sheen 2018; Brunkard 2020; Peixoto*et al.* 2021).

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 organized into a timeline. At first, electrons build up along the electron transport chain, and reduction of Q_a 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_6f 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 signaling may match photosynthesis to growth without explicit TPU limitations.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

DATA AVAILABILITY

All data are available upon request to the corresponding author.

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Figures

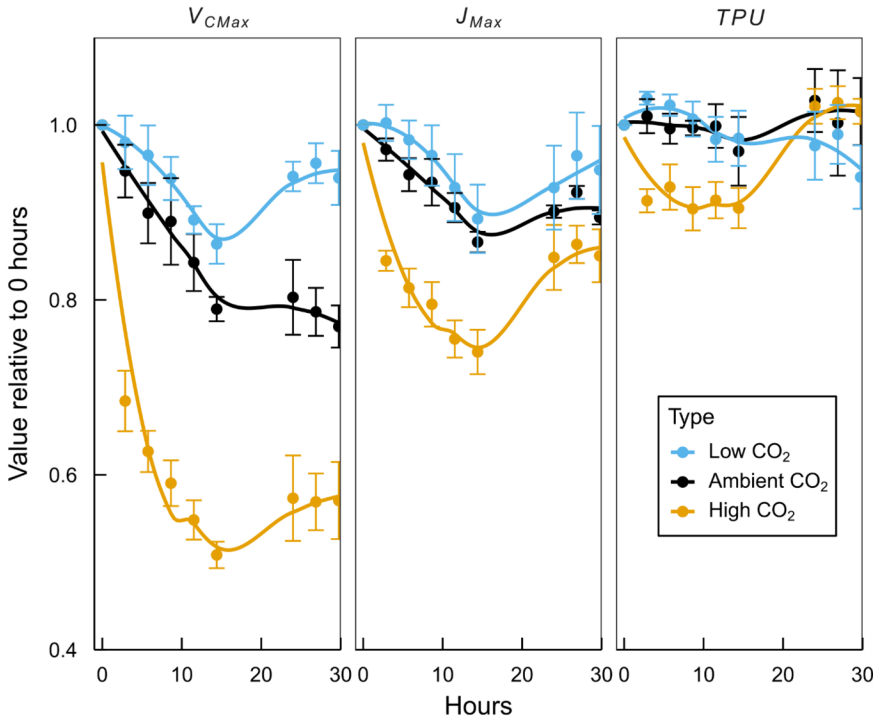

Figure 1: Plants were exposed to elevated (1500 ppm) ambient (400 ppm) or low (150 ppm) CO_2 for 30 h, including an 8-hour dark period during the typical night hours, with A/C_icurves performed every 2.5 hours. The A/C_i curves were fit according to Gregory *et al.* , (2021) and the three primary fit parameters, V_{cmax} , J , andTPU relative to an A/C_i curve run before treatment began are plotted.

Figure 2

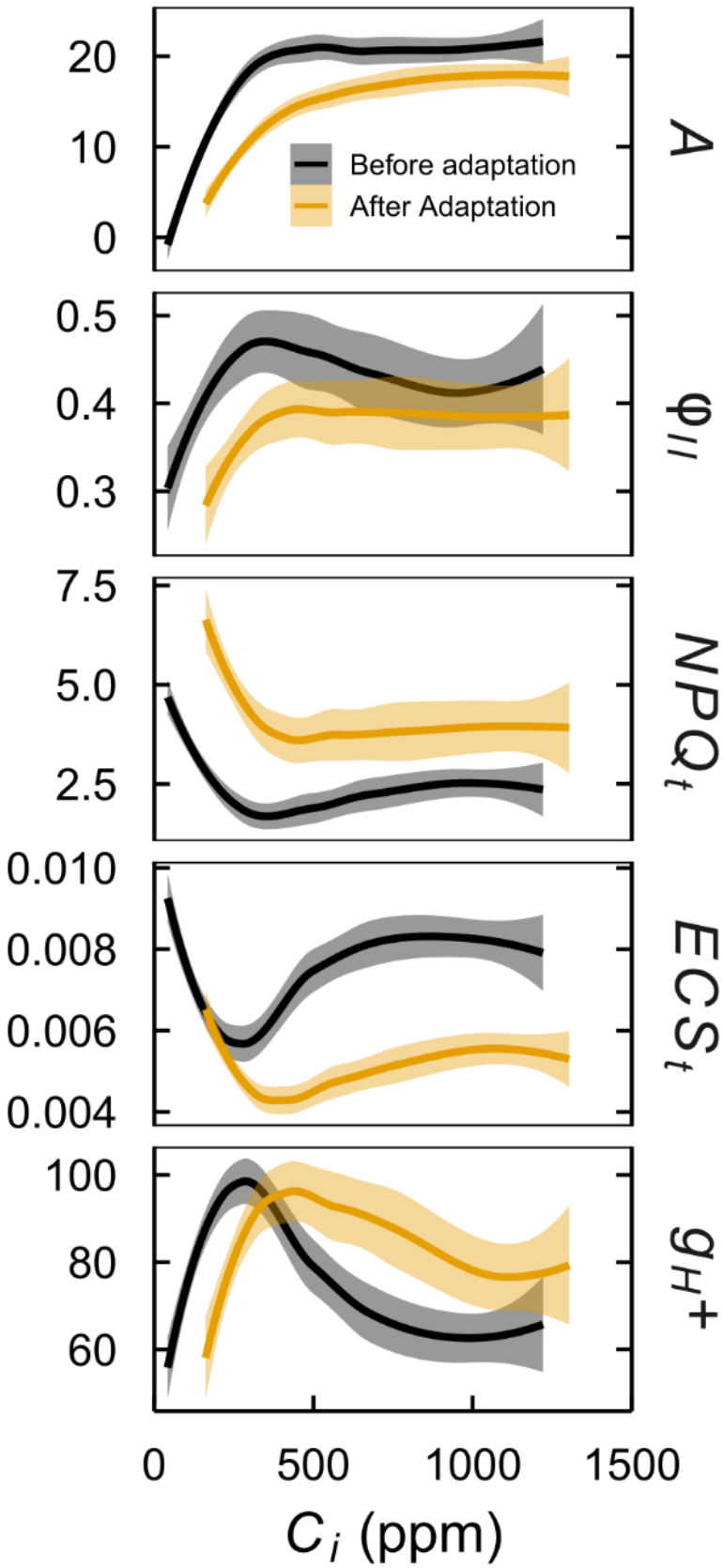

Figure 2: CO_2 assimilation and optical measurements from an A/C_i curve before and after a 30 h elevated CO_2 treatment. After 30 h in elevated CO_2, parameters show acclimation to TPU-limiting conditions, including reduced response of assimilation,φ_{II} , NPQ_t , andECS_t to increasing CO_2. The clouds are LOESS fitting (LOcal Estimation of Scatterplot Smoothing) 95% CI n=5.

Figure 3a

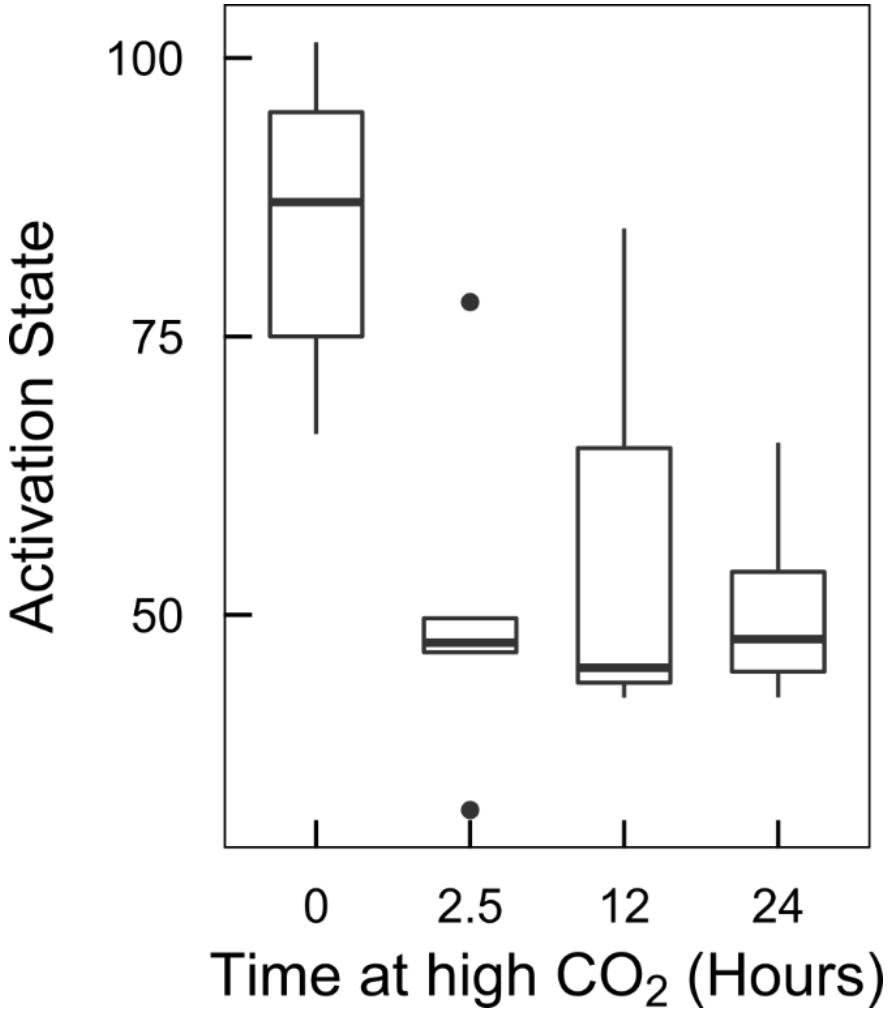

Figure 3b

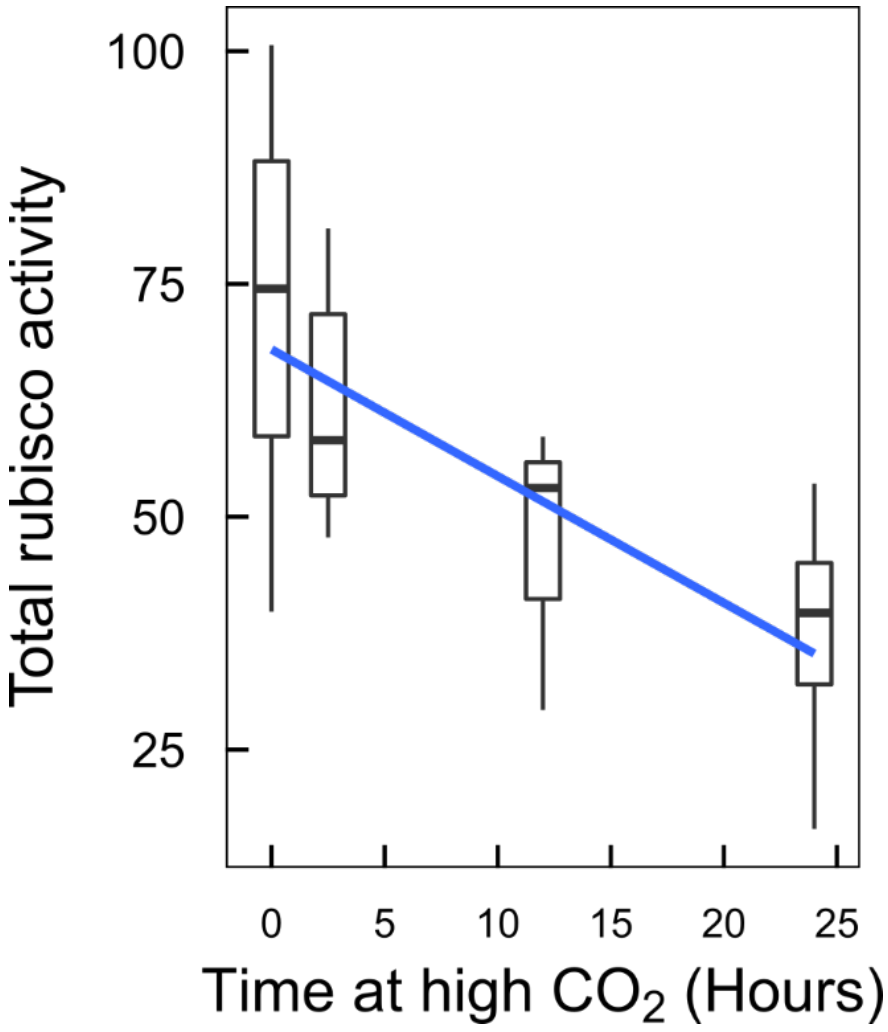

Figure 3c

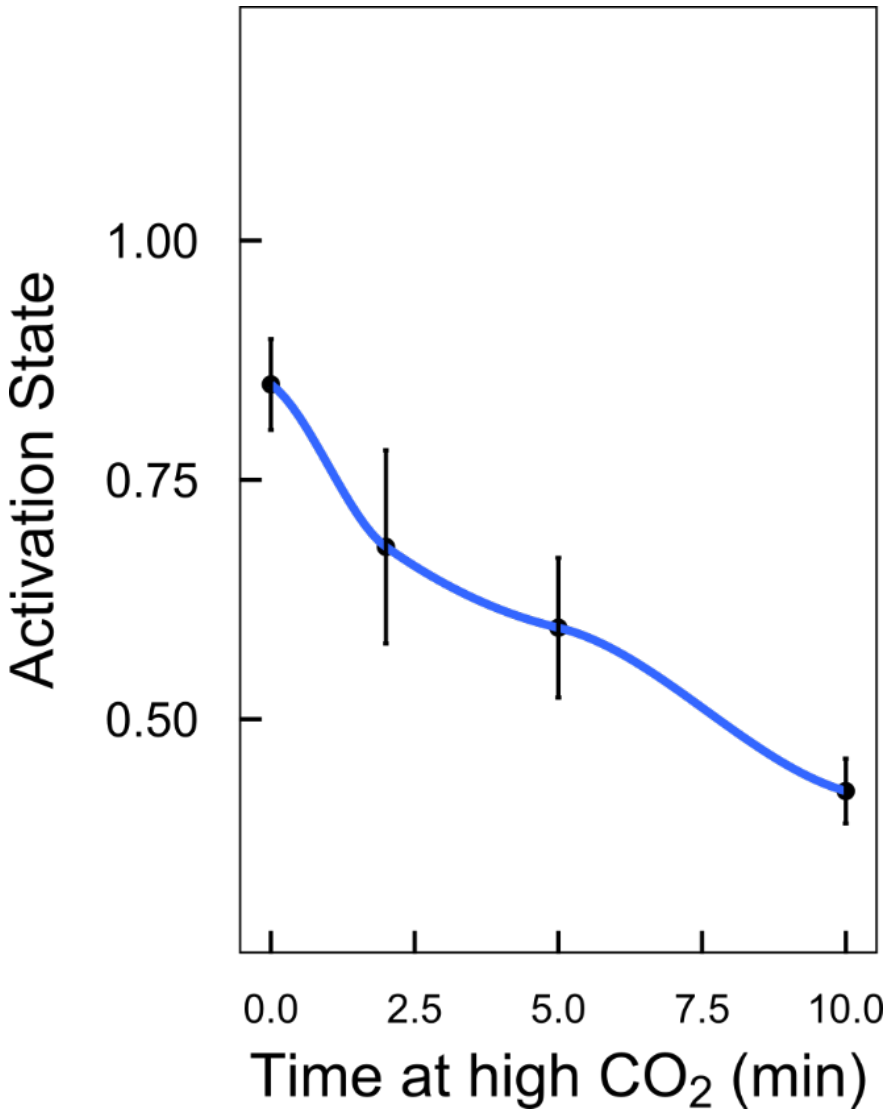

Figure 3d

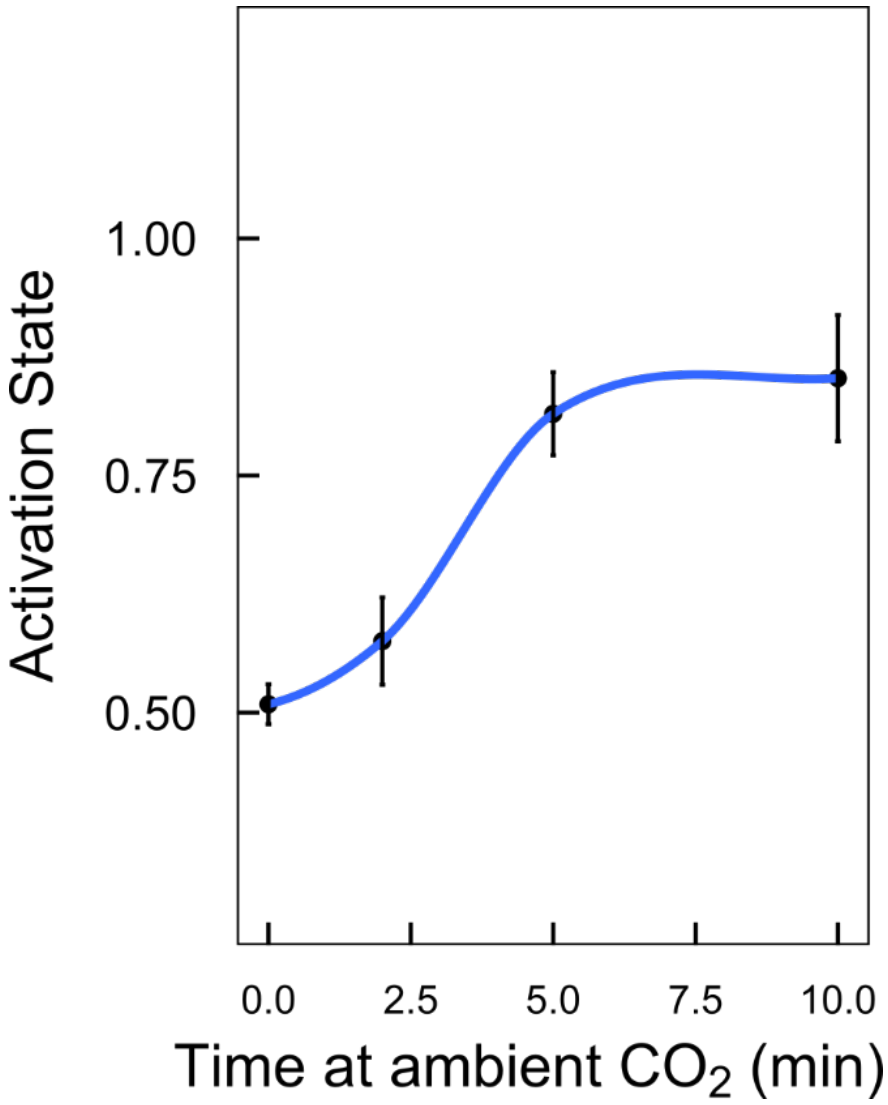

Figure 3: TPU limitation causes reduced rubisco activation state that persists for an extended period. Rubisco activation state remains low over the course of adaptation (a), and the total rubisco activity declines (b). Rubisco activation state decreases quickly after adding CO_2 initially (c) and remains recoverable over 10 min for at least the first several hours (d).

Figure 4

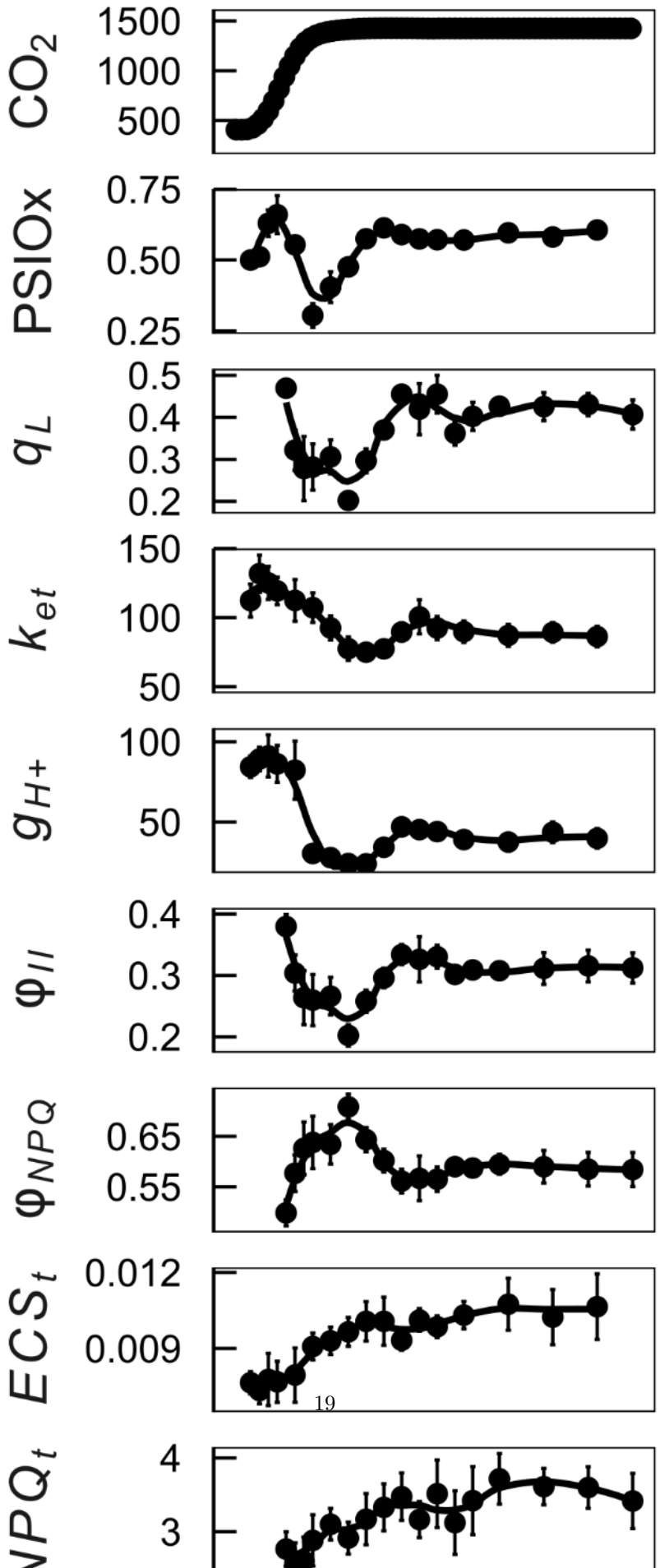

Figure 4: Plants are given a spike in CO_2, which induces oscillations in electron transport. In the first phase, photosynthesis is unlimited by TPU and PSI becomes more oxidized by the addition of extra CO_2, while proton conductivity remains high. The second phase (blue) is the earliest effect of TPU limitation and is primarily described by PSI and Q_a quickly become reduced (measured as PSI ox and q_L), along with constriction of proton flow across the thylakoid membrane (g_{H+}) and electron flow to PSI (k_{et}) blue-shaded region). The third phase (green) begins when slower regulations, which depend on proton-motive force (measured as ECS_t), energy dependent quenching (NPQ_t) and photoprotection at cytochrome *b6f* complex, relieve reduction of the electron transport chain. Finally, the electron transport chain enters a new steady-state (red).

Figure 5

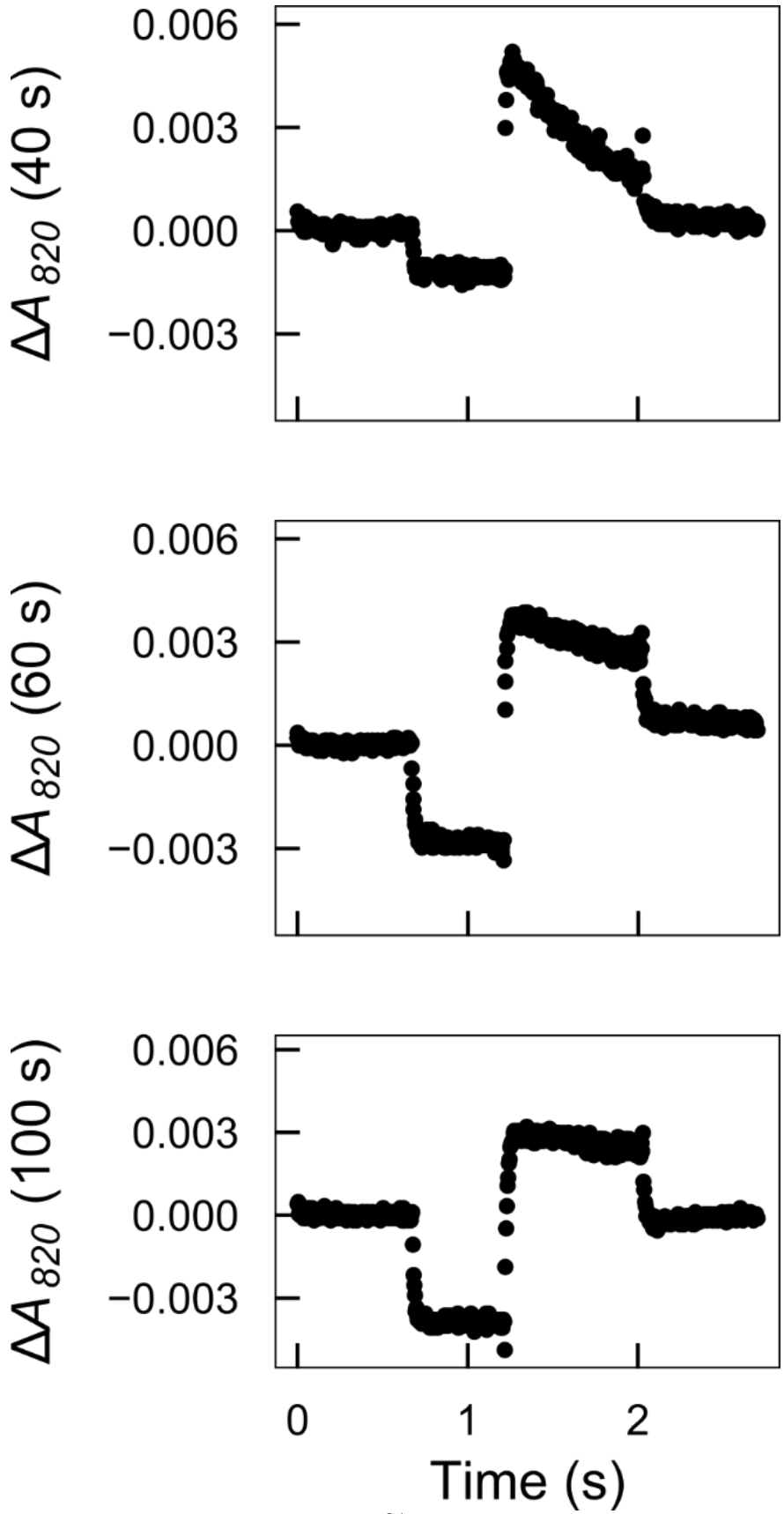

Figure 5: Three traces of PSI measurements from oscillations in PSI reduction induced by spike in CO_2, which demonstrate varying levels of re-reduction during saturating flashes. Typically, a saturating flash should fully oxidize PSI, but kinetics in electron transport can change this. (a) Extreme re-reduction of PSI can be seen during a saturation flash when PSI is most reduced, 40 s after beginning an elevated CO_2 pulse. This demonstrates a high level of PSI-acceptor side limitation. (b) Less re-reduction of PSI during a saturation flash is seen when PSI is less limited by electron acceptors 60 s after beginning a CO_2 pulse. (c) After returning to a new steady-state 100 s after beginning an elevated CO_2pulse, PSI acceptor-side limitation is much diminished, and PSI re-reduction is minimal.

Figure 6

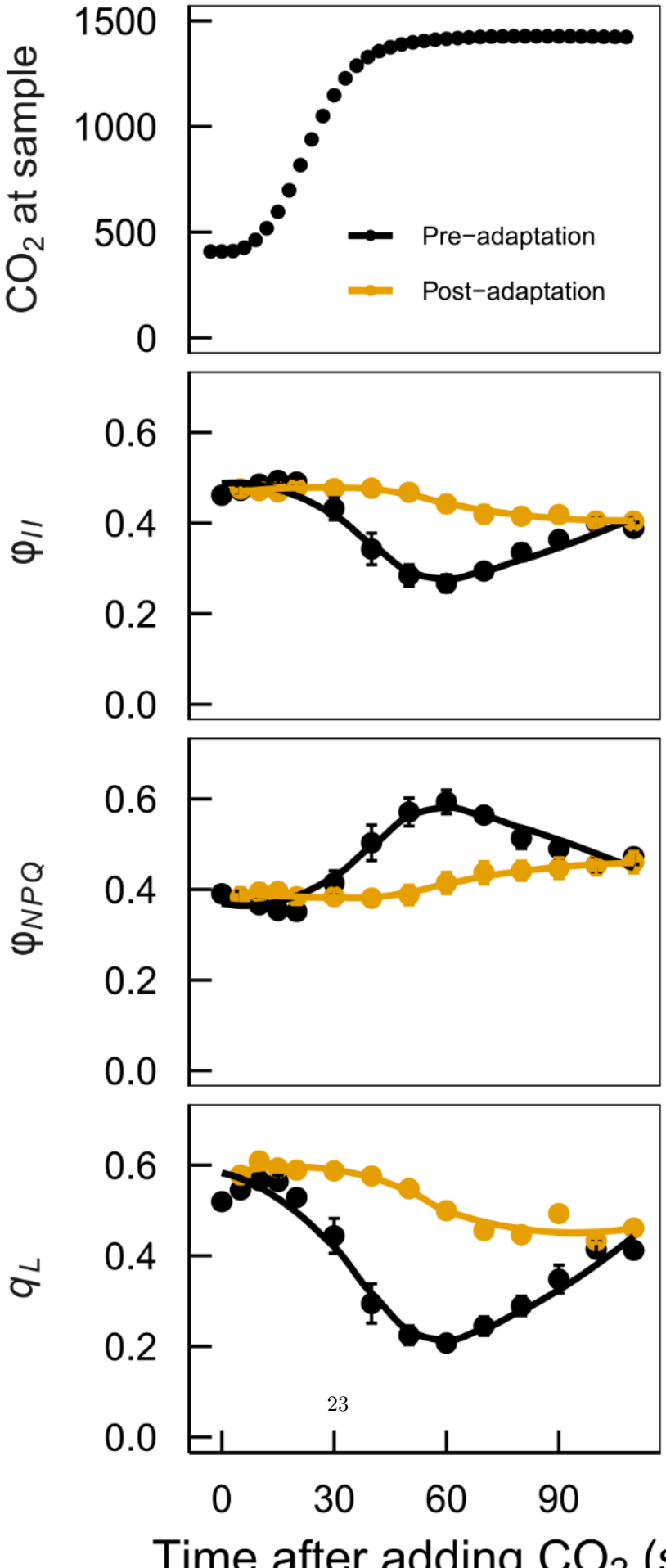

Figure 6: Oscillations are not seen following a spike in CO_2 in plants that have acclimated to elevated CO_2 for 30 h. The hallmark reduction of Q_a, measured here as q_L , is not seen, and so more energy is not diverted into non-photochemical quenching ($\varphi_{NПX}$).

Figure Legends

Figure 1: Plants were exposed to elevated (1500 ppm) ambient (400 ppm) or low (150 ppm) CO_2 for 30 h, including an 8-hour dark period during the typical night hours, with A/C_icurves performed every 2.5 hours. The A/C_i curves were fit according to Gregory *et al.* , (2021) and the three primary fit parameters, V_{cmax} , J , andTPU relative to an A/C_i curve run before treatment began are plotted.

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