

The Impacts of Fluctuating Light on Crop Performance¹

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Rapidly changing light conditions can reduce carbon gain and productivity in field crops because photosynthetic responses to light fluctuations are not instantaneous. Plant responses to fluctuating light occur across levels of organizational complexity from entire canopies to the biochemistry of a single reaction and across orders of magnitude of time. Although light availability and variation at the top of the canopy are largely dependent on the solar angle and degree of cloudiness, lower crop canopies rely more heavily on light in the form of sunflecks, the quantity of which depends mostly on canopy structure but also may be affected by wind. The ability of leaf photosynthesis to respond rapidly to these variations in light intensity is restricted by the relatively slow opening/closing of stomata, activation/deactivation of C_3 cycle enzymes, and up-regulation/down-regulation of photoprotective processes. The metabolic complexity of C_4 photosynthesis creates the apparently contradictory possibilities that C_4 photosynthesis may be both more and less resilient than C_3 to dynamic light regimes, depending on the frequency at which these light fluctuations occur. We review the current understanding of the underlying mechanisms of these limitations to photosynthesis in fluctuating light that have shown promise in improving the response times of photosynthesis-related processes to changes in light intensity.

As sessile organisms, plants must respond to dynamically and rapidly changing environmental conditions. Light intensity is the most dynamic condition to which plants must respond and can change at time scales from a season to less than 1 s (Assmann and Wang, 2001). The maximum amount of light incident at any point in time for a given area of ground is determined seasonally (Fig. 1A), but within seasonal variation, there is also variation as a function of the time of day from complete darkness to full sunlight (Fig. 1B). Clouds can substantially reduce incident light, and their intermittency introduces dynamic intensity changes (Fig. 1B). Atmospheric aerosols, which are a complex and dynamic mixture of solid and liquid particles from natural and anthropogenic sources, also can interact with the Earth's radiation budget and climate

ADVANCES

- Previously used steady-state conditions for measurements and modeling are inadequate for evaluating crop photosynthesis and productivity in field conditions. The limitations to crop photosynthetic efficiency in fluctuating light present multiple opportunities for study and potentially substantial improvement in productivity.
- Manipulating regulatory enzymes, such as Rca and the enzymes involved in the xanthophyll cycle, present potential promising candidates for improving photosynthesis regulation in fluctuating light.
- Unsuccessful attempts to manipulate stomatal kinetics through single-gene mutations and recent improvements in NPQ relaxation via simultaneous manipulation of several pathway genes imply that single-gene targets are less plausible for improving photosynthetic kinetics in dynamic light conditions.
- C_4 plants are more sensitive than C_3 plants to dynamic light conditions, possibly due to the additional complexity of coordinating the bundle sheath C_3 and mesophyll C_4 cycles, but there is theoretical evidence that C_4 plants may be more resilient under certain frequencies of light fluctuation due to the buffering capacity of transport metabolites.

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directly by reflecting light back into space and indirectly by changing how the clouds reflect and absorb sunlight. These sources of variation in incident light upon a canopy can have a large impact on canopy photosynthesis because the upper canopy drives ~75% of crop canopy carbon gain (Long et al., 2006). While the amount of sunlight reaching lower leaves within a canopy depends on many factors, sunflecks provide the majority of light in the lower canopy (Fig. 1C). Despite the transient nature of sunflecks, they can provide up to 90% of the available light in lower layers of dense canopies, highlighting the importance of harnessing these rapid fluctuations of light for canopy productivity (Chazdon, 1988; Knapp and Smith, 1989; Pearcy, 1990; Chazdon and Pearcy, 1991; Way and Pearcy, 2012).

The importance of within-canopy photosynthetic performance on crop photosynthetic performance has been long recognized (Ort and Baker, 1988), and there is a growing realization of the contribution from within-canopy fluctuating light on crop productivity (Murchie et al., 2009; Lawson et al., 2012). For example, concurrent to many reviews focusing on the effects of sunflecks on forest understories (Chazdon, 1988; Chazdon and Pearcy, 1991; Pearcy and Pfitsch, 1995; Way and Pearcy, 2012), there have been advances in examining the same illumination dynamics in major crop species such as soybean (*Glycine max*; Pearcy et al., 1990, 1997), rice (*Oryza sativa*; Nishimura et al., 1998, 2000), and maize (*Zea mays*; Wang et al., 2008) using experimental and modeling approaches. Given the importance of dynamic light in crop canopies, various light-sensitive factors have been identified as targets to improve crop photosynthesis and productivity in fluctuating light conditions. For example, changes in canopy and leaf morphology can improve sunfleck abundance and distribution throughout canopy layers, while stomatal opening/closing kinetics determine carbon supply and water use during these transient periods of light. Delays in C_3 cycle enzyme activation reduce the efficiency with which available CO_2 is used during transitions to high light, and the slow relaxation of photoprotection lowers light use efficiency upon transitions to low light. The coordination of C_3 and C_4 cycles in C_4 crops presents an additional layer of complexity during dynamic light conditions. Although large metabolite pools may aid C_4 photosynthesis in acclimating rapidly to fluctuating light, some evidence suggests the contrary. Thus, the mechanisms of these processes are currently being studied in more detail under fluctuating light conditions and are the focus of this Update.

SEEING THE SUN THROUGH THE TREES

The degree of variation in light intensity incident on a given leaf is strongly dependent on the structure of the canopy around it, especially within the dense canopy structures typical of mature crop stands. Light variation within the canopy is affected by leaf shading by other leaves, which varies rapidly (Percy et al., 1990) and

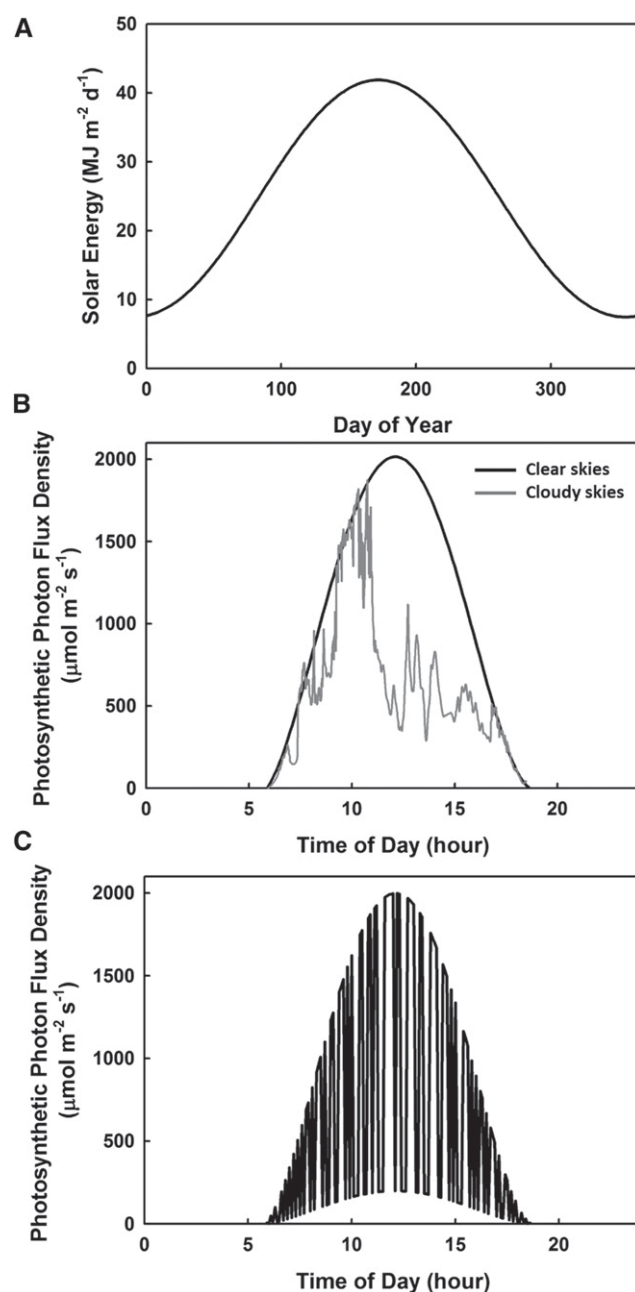


Figure 1. Dynamics of light intensity above and within crop canopies. A, Maximum solar energy incident upon a canopy over the course of a year at 50°N. B, Light intensity at the top of a canopy on a clear sunny day (black line) and on a day with intermittent cloud cover (gray line). C, Light reaching a midcanopy leaf on a clear sunny day. Graphs are based on SURFRAD data from Bondville, Illinois (40°N latitude; B), and Zhu et al. (2004; C).

depends on a host of environmental, physiological, and morphological factors. As compared with forests, crop canopies have steeper angles of light penetration due to their short, dense canopies, resulting in fewer partially shaded regions. Thus, a large proportion of within-canopy fluctuating illumination comes from high-intensity,

lower-canopy sunflecks, especially in crops, such as soybean, as compared with forest canopies (Pearcy et al., 1990). Although the total number of sunflecks may decrease as canopies become denser, sunflecks still contribute a large percentage of total light intercepted by the canopy, even with relatively dense leaf area index values greater than five. Leaf arrangement affects the abundance of sunflecks in lower canopies. The quantity of sunflecks decreases with greater leaf clumping in modeled, artificial, and experimentally manipulated tree and crop canopies under a fixed leaf area index (Chen and Black, 1992).

Leaf angle also can determine sunfleck frequency depending on what proportion of incoming light is diffuse. For example, leaf angle and orientation in rice influences the dynamic nature of incident light under sunny but not overcast diffuse light conditions (Nishimura et al., 1998). An increased proportion of diffuse light allows the penetration of light to deeper layers of a crop canopy, which improves light use efficiency and photosynthesis at the canopy level (Sinclair and Muchow, 1999; Roderick et al., 2001; Li et al., 2014). Diffuse light also reduces the temporal variation in light intensity, thus reducing the relative contribution of sunflecks to canopy photosynthesis and dampening the effects of fluctuating light on canopies (Pearcy and Pfitsch, 1995; Way and Pearcy, 2012; Li et al., 2016). The relative proportion of light incident as either sunflecks or diffuse light varies depending on canopy structure and depth. In layers of a soybean canopy where sunflecks occur, sunflecks can account for 20% to 93% of the total available light on cloudless days, with the remainder being diffuse (Pearcy et al., 1990). However, there is evidence that radiation incident on Earth's surface is becoming more diffuse as a result of anthropogenic aerosol emission and changes in weather patterns, resulting in a global dimming of global surface radiation and possible changes to primary productivity (Mercado et al., 2009; Wild, 2009). Therefore, while diffuse light is important to overall canopy photosynthesis, it is not a focus of this review on fluctuating light in crop canopies other than to highlight its general importance and mention that it serves to dampen the impact of sunflecks.

It has long been recognized that wind plays an integral role in driving within-canopy light variation, but its effects are difficult to model, given the static and averaging nature of previous canopy models when representing plant structure and carbon assimilation. Recent advances in ray-tracing algorithms facilitated by improved imaging and computational abilities have enabled models with greater ability to account for the actual structure of a crop canopy and more precise localization of light regimes (Song et al., 2013; Burgess et al., 2015). These models have been leveraged to produce a representation of the impact of wind speed, variability, and direction on intercanopy radiation regimes and the net assimilation of a wheat canopy (Burgess et al., 2016). This work reveals that an increase in light penetration into the canopy due to wind-driven canopy movement can increase total canopy carbon

gain by up to 17% and raises interesting possibilities for the improvement of canopy photosynthesis through canopy structural modifications.

Individual leaf morphology, diurnal response, and ultrastructure also can impact the response of crop plants to variable light regimes. For example, long-term exposure to either high or low light affects aspects of leaf development and morphology, such as size, thickness, and shape, that impact light absorption (Boardman, 1977). Diurnal changes in light can affect leaf movement and orientation, allowing leaves to track the movements of the sun across the sky or avoid direct sunlight in times of stress (Raven, 1989; Ehleringer and Forseth, 1990). In a matter of minutes following an alteration in light intensity, chloroplasts rearrange within cells, from the upper surfaces of the cell in low light to the sides of the cell in high light (Wada, 2013). These effects and responses are important considerations for how crop plants respond to seasonal, diurnal, and transient changes in light intensity at the leaf and canopy levels, with biochemical considerations constituting the focus of the remaining discussion.

THE PHYSIOLOGICAL POLITICS OF STOMATA

Stomata present the initial limitation in both C_3 and C_4 plants by controlling the entry of CO_2 into the leaf and, therefore, the substrate supply for photosynthesis. Stomata also are responsible for balancing CO_2 uptake for photosynthesis with water loss from the leaf through transpiration. Stomatal closure conserves water when there is no need for CO_2 entry into the leaf for photosynthesis, but stomata must open to provide the CO_2 necessary for the carbon reduction cycle (Lawson and Blatt, 2014). The opening and closing of stomata are regulated by guard cells, the cells that surround the stomatal pore and regulate the pore aperture. Blue and red light-mediated processes result in an influx of solute and, therefore, water into the guard cells, causing an increase in turgor that opens the stomata. When solute concentrations decline, guard cells lose water, resulting in stomata closure. Other external signals also are related to stomata aperture control, including CO_2 concentration, humidity, temperature, and abscisic acid concentrations (Lawson, 2009).

While stomatal conductance and photosynthesis are generally well coordinated in steady-state conditions (Wong et al., 1979; Farquhar and Sharkey, 1982), the lag in photosynthetic response during transitions from low to high light (Fig. 2A) often is due to an insufficient supply of CO_2 needed for the carbon cycle. This is because the opening of stomata occurs at a much slower rate than the initial up-regulation of photosynthetic electron transport (Fig. 2B). In times of rapidly changing light conditions, this limitation can represent a substantial inefficiency in photosynthesis and, therefore, crop productivity. A recent study by McAusland et al. (2016) measured a 10% to 15% limitation to photosynthesis across several C_3 and C_4 crop species during

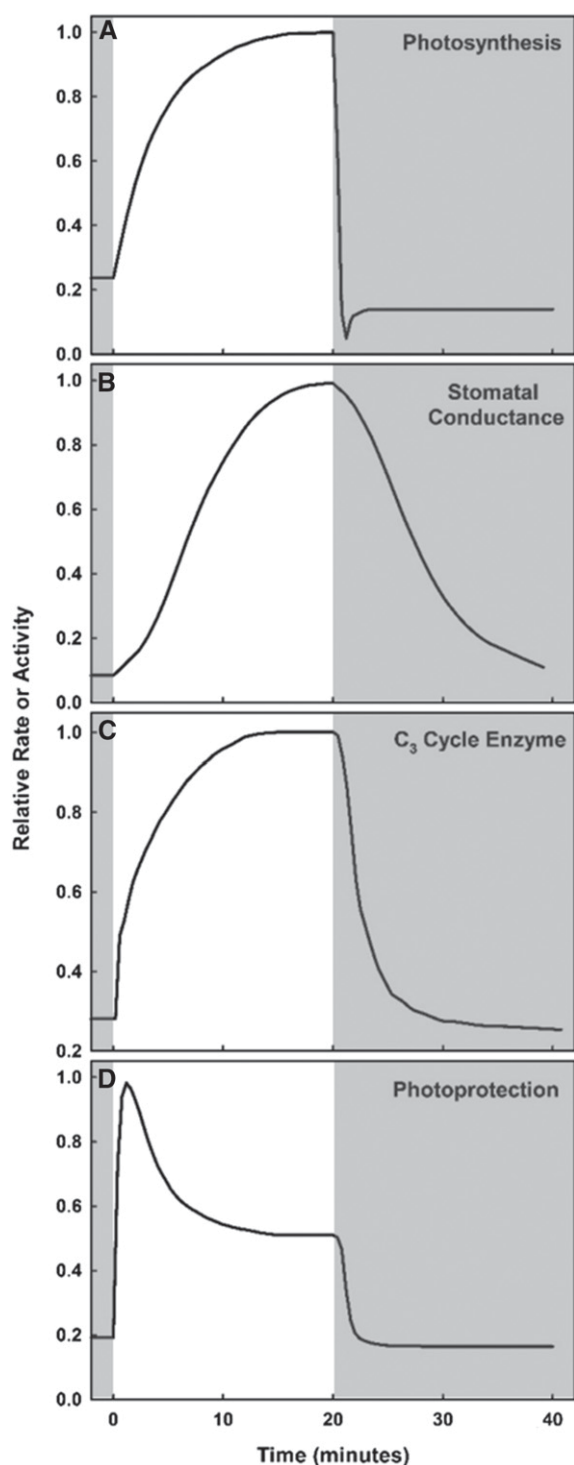


Figure 2. Schematic showing the relative induction and relaxation rates of photosynthesis-related processes during changes in light intensity. Relative rates of photosynthesis (A), stomatal conductance (B), C_3 cycle enzymes (C), and NPQ (D) are shown as functions of time during transitions from low light (gray background) to high light (white background) and from high light to low light. Curves are based on data from McAusland et al. (2016), Sassenrath-Cole and Percy (1994), and Kromdijk et al. (2016).

the time it took leaves to reach steady-state conditions upon transfer from low light to high light. However, the limitation to carbon assimilation by stomatal conductance during transitions from low to high light can vary depending on the initial stomatal conductance in low light. If stomatal conductance is greater in low light, which might occur if water is not limiting (Lawson et al., 2012; Way and Percy, 2012) and was the case in the McAusland et al. (2016) study, stomatal limitation is likely to be less during the transition, whereas lower stomatal conductance in low light, which might be evident in water-limited conditions (Knapp and Smith, 1989), likely leads to much higher stomatal limitation to photosynthetic recovery. It should be noted that, in fluctuating light, greater stomatal conductance at low light also could alleviate temperature stress via evaporative cooling upon sudden exposure to high light intensity (Schymanski et al., 2013) but at the expense of water use efficiency.

A sluggish stomatal response when leaves are rapidly shaded by a cloud or overhead foliage also can cause excessive water losses due to a lack of coordination between assimilatory demand for CO_2 and stomatal conductance. Thus, when stomata do not close rapidly enough, water is lost disproportionately relative to carbon gained, which reduces water use efficiency during the transition (Lawson and Blatt, 2014; McAusland et al., 2016). Upon low to high light transitions in some species, stomata conductance continues to climb, even after maximum rates of photosynthesis have been reached; thus, these species overshoot the rate of stomatal conductance that achieves maximum photosynthesis, which also reduces water use efficiency (McAusland et al., 2016). Some C_3 crops bred for high photosynthesis rates display lower water use efficiency in fluctuating light (McAusland et al., 2016), likely because selection for yield in non-water-limiting conditions does not penalize accessions with inferior water use efficiencies (Fischer et al., 1998; Koester et al., 2016). Given that crop productivity and yield often are water limited and are likely to become more so (Ort and Long, 2014), improving stomatal closure kinetics and regulation in response to fluctuating light regimes could substantially improve canopy water use efficiency and yield.

Careful coordination of stomatal kinetics and photosynthesis are needed to both ensure CO_2 availability for photosynthesis and limit water loss, especially for C_3 crops. While some studies suggest that many small stomata could achieve this end (Drake et al., 2013; Raven, 2014), other research suggests that stomata type and biochemistry may be more influential. A smaller change in turgor can have larger effects on stomatal aperture in dumbbell- versus elliptical-shaped guard cells, leading to more rapid opening/closing kinetics (Hetherington and Woodward, 2003; McAusland et al., 2016). In addition, equal and opposite transfer of turgor from subsidiary epidermal cells to guard cells during opening and closing, rather than constant pressure in the subsidiary cells, not only enhances stomatal

aperture by allowing displacement of the subsidiary cell but also increases the rate of stomatal movement (Franks and Farquhar, 2007; Raissig et al., 2017). The rate of solute transport into and out of guard cells also presents potential targets for modification to obtain more rapid opening upon transitions to high or low light (Lawson and Blatt, 2014; Wang et al., 2014b), but the intuitive solution of increasing the ratio of individual ion channels per surface area often has the opposite effect (Wang et al., 2014b, 2014c). However, modeling suggests that manipulating the gating of these ion channels may have more favorable consequences on stomatal kinetics (Wang et al., 2014b).

Despite the complexity and lack of established approaches to manipulate stomatal kinetics, recent studies have shown that variation exists within and among certain species that could guide future engineering efforts or enable optimization by selective breeding. McAusland et al. (2016) showed substantial variation in stomatal responses across 13 crop species, and measurements conducted by Qu et al. (2016) showed variation in stomatal kinetics among 204 rice accessions. Noncrop species also may provide clues for ways in which to improve stomatal kinetics. For example, unlike the delayed increase in stomatal conductance compared with photosynthesis seen in several crops (McAusland et al., 2016), the stomatal response to increased light of the C_4 shade-tolerant *Microstegium vimineum* is faster than the photosynthetic response following low-light acclimation (Horton and Neufeld, 1998). Other traits may be important to study in crops, such as the degree of anisohydry, or the tendency of stomata to remain open for CO_2 entry into the leaf despite a decline in leaf water content, across species, as stomatal kinetics are more rapid in tree species with more anisohydric tendencies (Meinzer et al., 2017). In addition, variation could be explained by differences in the speed of signaling components driving the mechanistic changes. Therefore, identifying the sources of variation in stomatal kinetics will be crucial for mechanistic understanding and further engineering efforts to increase the rate and coordination of stomatal opening and closing with light intensity.

Mesophyll conductance presents an additional limitation to carbon supply to the chloroplast during fluctuating light. Mesophyll conductance varies within minutes when leaves are exposed to changes in light, temperature, and CO_2 concentration (Flexas et al., 2007, 2008; Tholen et al., 2008; Evans and von Caemmerer, 2013), and more recent research has shown reductions in mesophyll conductance when plants are grown in fluctuating light conditions (Huang et al., 2015; Violet-Chabrand et al., 2017). Although the mechanisms for these reductions are not yet understood, aquaporin levels, carbonic anhydrase concentration, and leaf and cell anatomy may contribute to variation in mesophyll conductance (Flexas et al., 2012). In a recent study, growth in fluctuating light resulted in thinner palisade and spongy mesophyll layers and altered cell shape as compared with steady-light conditions

(Violet-Chabrand et al., 2017), but a direct relationship to overall mesophyll conductance was not investigated. Thus, elucidating the mechanisms of mesophyll conductance also is necessary for engineering to ensure sufficient carbon supply to photosynthesis during fluctuating light conditions.

THE CONTROL OF CARBON REDUCTION ENZYMES IN THE FACE OF STOP-AND-GO TRAFFIC

While stomatal and mesophyll conductance kinetics determine the supply of CO_2 for photosynthesis during dynamic light conditions, the activation rate of C_3 cycle enzymes determines how rapidly available CO_2 is reduced to form sugars. Upon the transition from low light to high light, such as occurs when a shaded leaf is exposed to a sunfleck, electron transport responds almost instantaneously. However, the relatively slower induction of the carbon reduction cycle (Fig. 2C) limits photosynthesis, thereby limiting total carbon gain by the leaf and canopy. In tobacco (*Nicotiana tabacum*), the time needed to reach maximum stomatal conductance in response to light (~ 40 min; McAusland et al., 2016) is greater than the time needed to reach maximum Rubisco activation (~ 7 min; Hammond et al., 1998) and is likely more limiting in some situations. However, in conditions favoring higher initial stomatal conductance, such as the brief periods between high-frequency sunflecks when biochemical enzymes relax more quickly than stomatal conductance, or in high CO_2 concentrations, such as those present in the lower canopy, C_3 cycle enzyme regulation likely limits photosynthesis to a greater degree than stomatal conductance (Sassenrath-Cole and Pearcy, 1992; Tomimatsu and Tang, 2012; Graham et al., 2017).

While gene expression and protein synthesis of enzymes involved in carbon reduction determine enzyme concentrations and, therefore, activity at longer time scales, reversible posttranslational modifications are responsible for the activation/deactivation of these enzymes during the more rapid transitions between light and dark conditions or even due to more modest changes in light intensity. The key enzymes controlling the C_3 cycle during light intensity transitions include Rubisco, Rubisco activase (Rca), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Fru-1,6-bisphosphatase (FBPase), sedoheptulose-1,7-bisphosphatase (SBPase), and phosphoribulokinase (PRK).

Before Rubisco can catalyze the carboxylation reaction of photosynthesis, the enzyme must undergo a series of reactions to reach its activated state. A Lys residue in Rubisco must be carbamylated by CO_2 and then stabilized by Mg^{2+} before it combines ribulose-1,5-bisphosphate with CO_2 in photosynthesis (or oxygen in photorespiration). However, the activation process can be inhibited by the binding of sugar phosphates, such as ribulose-1,5-bisphosphate, to Rubisco's active site, which prevents carbamylation. Thus, a separate process is needed to free the Rubisco active site for

activation and is achieved through the activity of Rca. Rca hydrolyzes ATP to remove sugar phosphates from Rubisco, thus allowing Rubisco to proceed through the reactions necessary for full carbamylation and activation (Wang and Portis, 1992; Portis, 2003). But what activates Rca? In many species, there are two forms of Rca: a longer α -isoform, which contains two Cys residues that, when reduced by thioredoxin, render the enzyme less sensitive to inhibition by ADP and, when oxidized, render the enzyme more strongly inhibited by ADP; and a shorter β -isoform, which lacks this redox regulatory component. Therefore, as light levels increase, the ATP/ADP ratio and the redox poise of thioredoxin increase levels of activating Rca in species containing the α -isoform. However, this may not be the only mechanism for enabling Rca to function, as some species contain only the shorter β -isoform version of Rca, which is not directly redox regulated but responds to ATP/ADP ratios in a species-dependent manner and also is able to activate Rubisco in light (Portis, 2003; Portis et al., 2008).

Rubisco and its activation by Rca are important potential targets for crop improvement, especially with regard to photosynthesis during changes in the light environment (Parry et al., 2013; Carmo-Silva et al., 2015). While Rca quantity is not usually considered limiting to Rubisco activation unless reduced by more than 60%, these results only refer to steady-state conditions (Carmo-Silva et al., 2015). Under dynamic conditions, tobacco expressing antisense Rca constructs shows a linear response between Rca content and the initial slope of carbon fixation during the transition from low to high light under a broad range of Rca contents, indicating that Rca indeed limits the induction of carbon fixation (Hammond et al., 1998). However, the reduction in carbon assimilation upon the transition from high to low light is not sensitive to Rca content (Hammond et al., 1998). A modeling study by Mott and Woodrow (2000) shows that a greater allocation of resources to Rca at the expense of Rubisco benefits the induction time of photosynthesis in fluctuating light conditions. This greater investment in Rca is a typical characteristic of shade leaves (von Caemmerer and Quick, 2000), which ensures more efficient use of available light in the form of sunflecks (Percy, 1990). Experimental overexpression of Rca in rice increases Rubisco activation and the rate of photosynthesis induction upon the transition from low to high light (Fukayama et al., 2012; Yamori et al., 2012). However, overexpression of Rca in nonfluctuating light conditions leads to a decrease in total Rubisco content and a small reduction in net carbon assimilation (Fukayama et al., 2012), confirming that Rca is only limiting in fluctuating light conditions (Yamori et al., 2012). Modifying the regulation of Rca may present a more attractive option to increasing the rate of photosynthetic induction upon light transitions. In an Arabidopsis transformant containing only the non-redox-regulated Rca β -isoform, which is insensitive to physiologically relevant ATP/ADP ratios, Rca remains activated in low

light, resulting in an instantaneous activation of Rubisco upon the transition to high light. This leads to faster rates of photosynthetic induction, which is correlated with large increases in biomass in fluctuating light compared with nonfluctuating light conditions that are not seen in the wild type (Carmo-Silva and Salvucci, 2013). The presence of two Rca isoforms with different regulatory properties raises the question of why Rca is regulated at all if constant activation is beneficial for carbon assimilation. The answer may lie, at least in part, in the observation that some inhibitors of Rubisco, like carboxy-D-arabinitol 1-phosphate, bind Rubisco during dark periods and may help protect against proteolysis (Andralojc et al., 1994; Khan et al., 1999). Therefore, given the cost of Rubisco biosynthesis, efforts to improve net assimilation through constant Rubisco activation should examine the impact on Rubisco turnover as well. In addition, inactivation of Rca in the dark would limit unnecessary ATP hydrolysis to maintain Rca activation under non-carbon-fixing conditions.

As with the α -isoform of Rca, numerous other enzymes of the C_3 cycle are at least partially regulated by the ferredoxin-thioredoxin system, including the key enzymes involved in the carbon reduction cycle: GAPDH, FBPase, SBPase, and PRK. Enzyme activation by the chloroplast ferredoxin-thioredoxin system can take minutes for full activation when leaves are transferred from low to high light, thereby creating a lag in reaching full photosynthetic capacity (Sassenrath-Cole et al., 1994; Sassenrath-Cole and Percy, 1994). While there are several isoforms and subtypes of thioredoxins present in plant cells, recent studies have identified numerous thioredoxin proteins responsible for the activation of C_3 cycle enzymes in the light. Specific *f*-type thioredoxin proteins have been identified in the direct activation of C_3 cycle enzymes (Thormählen et al., 2013; Yoshida et al., 2015; Naranjo et al., 2016). In addition, *m*-type thioredoxins have been shown to have no effect on photosynthesis in steady-state conditions but alter photosynthetic efficiency in fluctuating light and, therefore, are essential in rapidly changing light conditions (Thormählen et al., 2017).

Additional systems have been shown to contribute to the regulation of carbon reduction enzymes in fluctuating light. The NADPH-thioredoxin reductase C (NTRC) pathway, which likely uses NADPH indirectly as an electron donor, plays a distinct but cooperative role with the ferredoxin-thioredoxin system in modulating chloroplast functions and in regulating FBPase and SBPase activity (Yoshida and Hisabori, 2016). The NTRC pathway also plays a key role in maintaining photosynthetic efficiency in fluctuating light by controlling the NADPH redox status of the stroma (Thormählen et al., 2017). Dissociation from large protein complexes also is involved in C_3 cycle enzyme activation. Although PRK and GAPDH are reduced directly by thioredoxins (Marri et al., 2009), they are not fully activated until dissociated from the complex they form with a small protein, CP12. The level of

dissociation correlates well with light levels, thus allowing a more rapid activation/deactivation response during dynamic light conditions than redox regulation alone (Howard et al., 2008; Marri et al., 2009). However, the presence of this complex is not universal across species (Howard et al., 2011), suggesting that the regulation of these enzymes may be even more intricate and diverse. Reversible phosphorylation also may contribute to enzyme regulation, as Rca phosphorylation seems to occur in dark but not light conditions (Kim et al., 2016). While the effects of phosphorylation on Rca activity are not known yet, this suggests a potential method of regulation for carbon reduction enzymes in addition to the thioredoxin-mediated regulation discussed above. Other posttranslational regulatory mechanisms directly affect Rubisco (Houtz et al., 2008), such as acetylation (Gao et al., 2016), but their effects in dynamic light have yet to be examined. As more of the key players are identified and the mechanisms by which they regulate carbon metabolism in rapidly changing light conditions are elucidated, they will likely be used to enhance the responsiveness of carbon reduction enzymes in fluctuating light.

IS PHOTOPROTECTION OVERPROTECTIVE?

The photosynthetic response to light is linear at low light intensities but becomes saturated as light increases. In many crops, light saturation occurs by 25% of full sunlight in the absence of other stresses (Long et al., 2006). When leaves are experiencing additional stress or light increases more rapidly than photochemical capacity (e.g. slow responses of stomata and C_3 cycle enzymes, as detailed above), light saturation may occur at even lower light levels (Fig. 2D). As a result, leaves at the top of the canopy experience excessive amounts of light during the majority of daylight hours (Ort, 2001). When absorbed light is in excess of photosynthetic capacity, it has the potential to damage photosynthetic proteins and membranes. Thus, plants have evolved various photoprotective mechanisms, the most universal and important of which is called NPQ, which enables chloroplasts to safely dissipate excess light as heat (Niyogi, 1999).

By engaging NPQ in high-light conditions, leaves achieve protection from photodamage without any cost to carbon gain. Photoprotection is beneficial to leaves at high light because it prevents irreversible damage to PSII that must be repaired through protein synthesis. However, when NPQ remains engaged under non-saturating light conditions, it limits carbon gain by lowering the maximum quantum yield of PSII and, thereby, the quantum efficiency of CO_2 assimilation (Long et al., 1994). While NPQ inductions can occur within a few seconds upon the transition to high light, the relaxation process during the transition from high light to low light is much slower and can take minutes to hours to occur. As a result, when plants are in a photoprotected state and light levels decline rapidly to

nonsaturating levels, some of the available light that could be safely harnessed for photochemical processes is dissipated as heat and, therefore, wasted (Fig. 2D). For crops grown in the field, fluctuations in light, such as when a leaf shades another leaf due the continuous diurnal change in solar azimuth or when a passing cloud occludes the sun, present opportunities for this inefficiency to significantly limit leaf and canopy carbon gain. In fact, it has been estimated that the slow relaxation of NPQ can limit the daily canopy carbon uptake of crops grown in the field by up to 32% (Zhu et al., 2004).

NPQ is composed of several processes involved with protecting the photosynthetic machinery from excess light. The most rapid of these processes is energy-dependent quenching, or q_E , which engages on the scale of seconds to minutes (Müller et al., 2001). q_E is initiated when protons accumulate in the lumen, thereby reducing the lumen pH. PsbS, a protein associated with PSII, senses the low pH and signals a conformation change in the LHCII protein (Li et al., 2000; Sachar et al., 2017). This conformational change occurs in concert with the xanthophyll cycle, through which violaxanthin is converted to zeaxanthin by violaxanthin deepoxidase in high light (Demmig-Adams, 1990). Quenching via state transitions, or q_T , also diverts excitation energy away from PSII, engages on a slightly longer time scale (5–10 min) than q_E , but accounts for a very small portion of NPQ in higher plants (Horton et al., 1996). In addition to enhancing q_E , the zeaxanthin pool size is associated with q_Z , or zeaxanthin-dependent quenching (Nilkens et al., 2010), which arises on a slower time scale (10–15 min) than q_E and q_T . q_I is the slowest onset process, requiring up to hours to appear, and often is associated with the accumulation of photodamage to PSII (Müller et al., 2001).

Due to its complexity, manipulations of single facets of NPQ have not been sufficient for improving the relaxation kinetics of NPQ while maintaining full capacity for CO_2 assimilation at high light. Overexpression of PsbS increases the induction and relaxation rates and increases the maximum capacity of q_E (Li et al., 2002a; Zia et al., 2011; Hubbart et al., 2012), representing a potential benefit to plant biomass under stressful light conditions (Li et al., 2002b) but at a cost to CO_2 assimilation under less stressful conditions, as q_E remains higher than needed (Hubbart et al., 2012). While an increase in zeaxanthin concentration can improve stress resistance (Johnson et al., 2007), it slows the kinetics of NPQ, demonstrating that zeaxanthin concentration is less important for induction/relaxation rates than the ratio of zeaxanthin to violaxanthin or the deepoxidation state of the xanthophyll cycle (Johnson et al., 2008). A lingering pool of zeaxanthin, which increases the deepoxidation state, may serve as a memory of previous high-light conditions (Murchie et al., 2009) that would delay the relaxation rate of NPQ in anticipation of another high-light event. Overexpression of an ion antiporter helps increase the lumen pH more quickly upon the transfer of leaves to low light, thus speeding

up the relaxation of qE (Armbruster et al., 2014). However, overexpression also leads to photoinhibitory effects at high light, thus requiring more sophisticated regulation of the protein for increasing relaxation kinetics. To overcome the limits of altering single processes within NPQ, a recent study by Kromdijk et al. (2016) used a multitarget approach to increase NPQ kinetics by transforming tobacco to overexpress PsbS, violaxanthin deepoxidase, and zeaxanthin epoxidase. The overexpression of violaxanthin deepoxidase and zeaxanthin epoxidase increased the kinetics of the xanthophyll cycle, which led to a lower deepoxidation state, while overexpression of Psbs maintained the amplitude of qE in high light when the deepoxidation state was low. When grown in chambers under fluctuating light, the transformants show increased photosynthetic efficiency and reduced average NPQ compared with the wild type. Moreover, this more complex approach of simultaneously altering the expression of multiple enzymes related to NPQ leads to a 15% increase in plant biomass when grown in field conditions under natural light fluctuations (Kromdijk et al., 2016). Since the mechanisms of NPQ are conserved across most plant species, altering NPQ kinetics will likely lead to increased yields in many other crops.

C₄ PHOTOSYNTHESIS: ADDING COMPLEXITY UNDER FLUCTUATING LIGHT

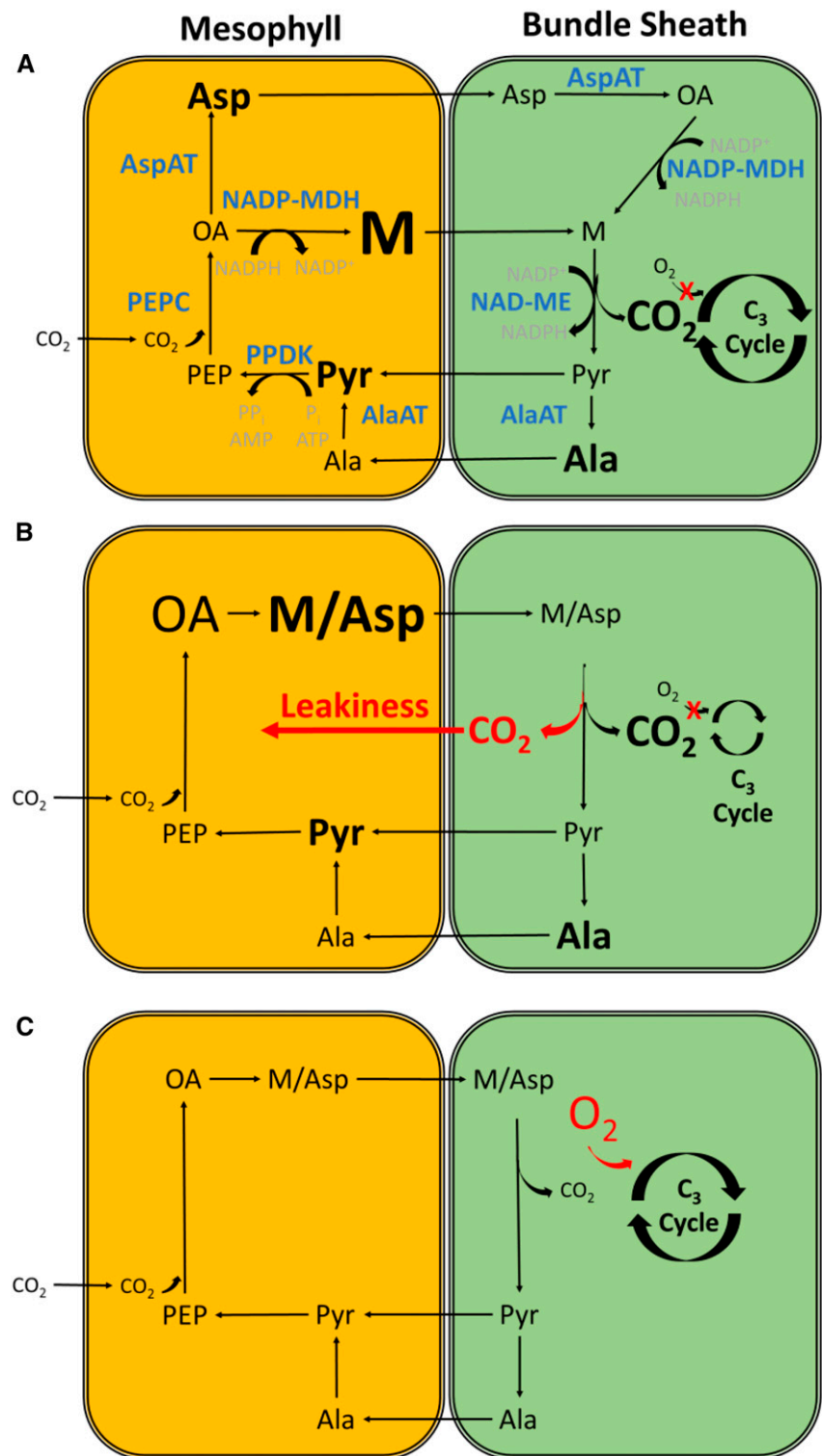
While the processes described above may apply to both C₃ and C₄ plants, the unique metabolism of C₄ plants may further impact their response to the fluctuating light of field conditions. Plants performing C₄ photosynthesis are among the most important crop species globally due to the high efficiency with which they can capture and reduce CO₂ with decreased water use and nitrogen investment in Rubisco. C₄ crops, such as maize, sugarcane (*Saccharum officinarum*), millet (*Pennisetum glaucum*), and sorghum (*Sorghum bicolor*), achieve higher carbon fixation rates by first carboxylating phosphoenolpyruvate (PEP) with atmospheric CO₂ to form oxaloacetate via PEP carboxylase (Fig. 3A). The carbon in oxaloacetate is then either reduced with NADPH into malate or converted into Asp before moving into the bundle sheath cell, where either substrate is decarboxylated. Thus, the concentration of CO₂ around Rubisco increases, which minimizes photorespiration in the coupled C₃ cycle. To maintain the cycle, reduced carbon must be transported from the bundle sheath cells back into the mesophyll cells to replace the carbon transported by the original C₄ carbonic acid. This return transport can occur directly via pyruvate produced following malate decarboxylation, via Ala produced from pyruvate through Ala aminotransferase, and/or via the shuttling of 3-phosphoglycerate from the C₃ cycle in the bundle sheath cells. PEP is then regenerated from pyruvate at the cost of ATP, which imposes an additional energetic cost to C₄ metabolism. C₄ species have traditionally been classified by the

different decarboxylating enzymes employed (i.e. NADP-ME, NAD-ME, and PEP-CK), but there is a growing consensus that C₄ plants like maize use multiple decarboxylation pathways in parallel (Pick et al., 2011; Arrivault et al., 2017). However, there are few examples of a crop plant exclusively using PEP-CK as a decarboxylation enzyme, which suggests that PEP-CK functions predominantly as a supplemental pathway supporting NADP-ME and NAD-ME decarboxylation (Wang et al., 2014a). Thus, only the NADP-ME and NAD-ME pathways are presented here (Fig. 3A).

The efficiency of C₄ photosynthesis depends on the coordination of C₄ carbonic acid production within the mesophyll cells with C₃ carbon reduction within the bundle sheath cells. There is ample theoretical and experimental evidence indicating that this coordination is impacted to some degree by variations in light regimes. For example, during high to low light transitions, accumulated malate and/or Asp are still available for decarboxylation in the bundle sheath cell, but there is insufficient photophosphorylation to generate the ATP necessary for C₃ carbon fixation. This could result in a transient overpumping of CO₂ into the bundle sheath cells and subsequent leaking of the CO₂ back into the mesophyll cells (Fig. 3B). This leakiness is energetically costly, since PEP must still be regenerated at the cost of ATP without the usual fixation of carbon (von Caemmerer and Furbank, 1999; Sage and McKown, 2006). Transient increases in leakiness are reported in many C₄ plants in response to low light shifts (Cousins et al., 2006, 2008; Kubásek et al., 2007; Tazoe et al., 2008; Pengelly et al., 2010), especially under light values lower than 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Kromdijk et al., 2010; Bellasio and Griffiths, 2014a), although it should be noted that the ¹³CO₂ discrimination assumptions used to interpret earlier reports can overstate the impact of transient light to leakiness (Ubierna et al., 2011, 2013; Kromdijk et al., 2014). While leakiness in the C₄ carbon pump results in inefficient carbon fixation and transient decreases in the quantum efficiency of C₄ carbon fixation, these losses appear small. But what about low to high light transitions?

The coordination of C₄ and C₃ cycles is complicated during the transition from low to high light due to the reliance of C₄ acid transport into the bundle sheath cell on concentration gradients (Fig. 3C). The transport of C₄ acids into bundle sheath cells is thought to occur mainly via diffusion along concentration gradients between the mesophyll and bundle sheath cells, which need to be a minimum of 5 to 10 mM to facilitate rapid transport in C₄ metabolism (Hatch and Osmond, 1976). Gradients between the two cell types have been determined experimentally for malate in maize and range between 6 and 88 mM (Leegood, 1985; Stitt and Heldt, 1985; Arrivault et al., 2017), indicating that large active C₄ acid pools are required. Therefore, during a low to high light transition, these large C₄ acid pools must accumulate to optimal values before the C₄ cycle and the C₃ cycle are synchronized. Before optimal pool sizes are established for a given light intensity, suboptimal CO₂

Figure 3. Schematic showing the relevant components of C_4 biochemistry and the theoretical impacts of light fluctuations on C_4 and C_3 cycle coordination. A, A simplified diagram shows two types of C_4 pathways, NADP-ME and NAD-ME. The PEP-CK pathway is absent. B, A high to low light transition results in a transient overpumping of CO_2 into and subsequent CO_2 leakage out of the bundle sheath. C, A low to high light transition results in higher rates of Rubisco oxygenation. The relative substrate size represents the concentration gradient between the mesophyll and bundle sheath cells based on measured data (see text). Metabolites are black, enzymes are blue, and cofactors are gray. Terms are abbreviated as follows: oxaloacetate (OA), malate (M), pyruvate (Pyr), PEP carboxylase (PEPC), NADP-malate dehydrogenase (NADP-MDH), Asp aminotransferase (AspAT), NAD malic enzyme (NAD-ME), Ala aminotransferase (AlaAT), and Pyr phosphate dikinase (PPDK).



concentrations near Rubisco would increase rates of oxygenation, leading to increased photorespiration and, thus, incurring the double costs of C_4 carbon pumping and C_3 photorespiration (Fig. 3C; de Veau and Burris, 1989; von Caemmerer and Furbank, 2003;

Sage and McKown, 2006; Bellasio and Griffiths, 2014b). The transport mechanisms returning C_3 acids into the mesophyll following decarboxylation in the bundle sheath cell are less clear. Ala and triose phosphate seem to require modest gradients between the mesophyll and

bundle sheath cell to facilitate return, but pyruvate shows small to even reverse gradients between the two cell types (Arrivault et al., 2017).

C₄ METABOLIC POOL SIZES IN FLUCTUATING LIGHT: A TALE OF TWO HYPOTHESES

The reliance of malate and Asp on gradient-driven transport and the pool sizes that enable this transport lead to two potentially contradictory hypotheses concerning the resilience of C₄ photosynthesis during low to high light transitions. One hypothesis, as suggested above, is that C₄ photosynthesis is more negatively impacted by fluctuating light than C₃ photosynthesis due to the transient incoordination between the C₃ and C₄ cycles occurring while transport gradients either collapse or reestablish. An alternative hypothesis suggests that the large pool sizes are beneficial from a photoprotective role. Under this hypothesis, C₄ metabolism can store excess harvested ATP and NADPH within the large pools of carbon-shuttling intermediates without having to resort to NPQ during transient increases in light energy capture, resulting in a higher quantum yield of photosynthesis and effectively using C₄ intermediates transiently as alternative electron acceptors or donors for the coupled C₃ cycle (Stitt and Zhu, 2014). The evidence for these contrasting hypotheses is discussed below.

There are several lines of experimental evidence indicating that C₄ photosynthesis is less resilient than C₃ photosynthesis to rapid fluctuations in light. For example, C₄ species relax their photosynthetic capacity more rapidly under decreasing light and take longer to reach high rates of photosynthesis under return to high light, resulting in a decreased ability of C₄ plants to rapidly reinduce photosynthesis in response to sunflecks (Chazdon and Pearcy, 1986; Horton and Neufeld, 1998; Sage and McKown, 2006). This is consistent with a requirement to first reestablish large malate or Asp pools upon illumination to achieve the optimal transport of carbon into the bundle sheath. While this may explain why examples of understory or shade-tolerant C₄ plants are few, it also suggests that the shaded regions within C₄ crop canopies would be less able to take advantage of rapidly fluctuating light regimes and frequent sunflecks that can occur in lower C₄ canopies (Tang et al., 1988). This relative difference also is seen in the slower response of maize leaves to simulated sunflecks as compared with soybean (Pons and Pearcy, 1992; Krall and Pearcy, 1993). A direct comparison of C₃ and C₄ responses to fluctuating light also has shown that plant biomass is reduced to a greater degree in C₄ plants (58% reduction) as compared with C₃ plants (30%–51% reduction) when grown under dynamic light conditions (Kubásek et al., 2013). Additionally, C₄ photosynthetic rates decrease during low-light periods in fluctuating light environments compared with steady-state light conditions, whereas C₃ photosynthetic rates increase, and dynamic light regimes

decrease the photochemical efficiency of C₄ plants more than C₃ plants due, in part, to C₄ leakiness, which also increases under dynamic light conditions (Kubásek et al., 2013).

Given the above observations, can the large metabolite pools involved in C₄ photosynthesis act to buffer energy supply and demand during fluctuating light in a way that confers any advantage to these crop species? While there are many potential advantages to the newly recognized flexibility of C₄ photosynthesis to utilize various metabolites with different energy balances (Stitt and Zhu, 2014), we focus here on malate transport, since we have the most data concerning its presence, specifically to estimate the total electron-buffering capacity of malate and its effective time scale. The total active malate pool was recently reported as $\sim 5 \mu\text{mol g}^{-1}$ fresh weight (Arrivault et al., 2017), which translates to $1,500 \mu\text{mol m}^{-2}$, assuming a fresh weight specific leaf area of 300 g m^{-2} . Since each malate carries the reductive power of two electrons and four electrons are required to reduce one CO₂ molecule, this malate pool contains enough reductive power to reduce $\sim 750 \mu\text{mol CO}_2 \text{ m}^{-2}$, or enough reductant to support the electron demands of carbon fixation occurring at a rate of $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$ for 15 s. Thus, the active pool of malate could contain a sufficient supply of reductant to buffer against transitions from high to low light, perhaps explaining why greater leakiness is not observed during these transitions, as discussed above. Of course, the estimate of 15 s is an upper boundary for the ability of the active malate pool to supply energy to the C₃ cycle during high to low light transitions, since malate transport would slow as pool sizes within the mesophyll and bundle sheath cell come into equilibrium and C₃ carbon fixation becomes limited by ATP availability.

Using the same logic, we can estimate the potential of C₄ metabolite pools to buffer against rapid shifts from low to high light by acting as electron sinks by determining the immediate capacity for carboxylation and reduction. The total active pool size of metabolites upstream of malate (only PEP, pyruvate, Ala, and 3-PGA; oxaloacetate was not determined) presented by Arrivault et al. (2017) is $3.6 \mu\text{mol g}^{-1}$ or $\sim 1,000 \mu\text{mol m}^{-2}$. This represents the capacity to provide alternative electron acceptors for $\sim 2,000$ electrons while the C₃ cycle activates and malate pools establish, or the capacity to provide 10 s of alternative electron acceptors, assuming an electron transport rate of $200 \text{ e}^- \text{ m}^{-2} \text{ s}^{-1}$ during a low to high light transition without having to initiate NPQ in the bundle sheath. Again, this represents an upper boundary to the possible buffering capacity, since all upstream metabolites would not be immediately transported into the bundle sheath and converted to oxaloacetate. Naturally, both calculations above depend upon the light conditions of Arrivault et al. (2017), which were moderate ($\sim 500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). However, these calculations serve as initial estimates of the impact that gradient formation and pool sizes can have on C₄ photosynthesis during light transitions.

Given the above calculations, the buffering capacity of C_4 photosynthesis during light fluctuations is expected to be limited to, at maximum, the first 10 to 15 s of a transition between light intensities. This is a much faster time scale than the minute time scales examined in $^{13}\text{CO}_2$ discrimination-based measurements of leakiness (Cousins et al., 2006, 2008; Kubásek et al., 2007; Tazoe et al., 2008; Pengelly et al., 2010) or growth analysis (Kubásek et al., 2013) discussed above but on the order of sunfleck light availability (Pons and Pearcy, 1992; Krall and Pearcy, 1993). Therefore, we argue that C_4 photosynthesis may be made both more and less resilient to dynamic light regimes, depending on the frequency at which these light fluctuations occur. We propose that a more systematic examination of the response of C_4 photosynthesis to a range of dynamic light frequencies would help resolve the interaction between C_4 photosynthesis and dynamic light.

CONCLUSION

To date, the majority of studies on crop photosynthesis have been performed in steady-state conditions, but more recent research has been trending toward exploring the responses of photosynthesis, especially in field-grown crops, to dynamic conditions. With this shift in emphasis has come the realization of the importance of the efficiency of photosynthesis in fluctuating light to the overall efficiency of canopy photosynthesis. The studies incorporating fluctuating light conditions have shown a lagging response of photosynthesis to both increasing and decreasing light

intensity, presenting a significant limitation to crop productivity that, at the same time, reveals opportunities for improving performance. Going forward, it is clearly important to incorporate measurements of non-steady-state photosynthesis in the experimental design of field experiments and into system models of crop growth and performance. This may be even more important when assessing photosynthetic performance under both dynamic light and future climate conditions. The effects of elevated CO_2 concentration on carbon gain in fluctuating light have been studied in limited species and show conflicting results (Tomimatsu and Tang, 2016; Kaiser et al., 2017), and the interaction of elevated CO_2 with other factors, such as increased temperature and altered precipitation patterns, remains to be studied. Therefore, research in artificial light environments must either attempt to simulate dynamic light conditions similar to those experienced in the field or acknowledge the potential biases that may be present in experimental results from steady-state light conditions (Violet-Chabrand et al., 2017).

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OUTSTANDING QUESTIONS

- Can the characteristics and/or mechanisms associated with more rapid stomatal kinetics in dumbbell-shaped guard cells be implemented into elliptical-shaped guard cells? How is mesophyll conductance regulated in fluctuating light? Can it be improved upon?
- Identification of the proteins involved in thioredox regulation of C_3 cycle enzymes is underway, but what is the mechanism of enzyme activation/deactivation? Is thioredoxin involved in the oxidation mechanism, and can these be improved upon for faster upregulation and downregulation of photosynthesis in dynamic light conditions?
- Are C_4 plants underutilizing the ability of intercellular metabolite pools to buffer photosynthesis during dynamic light conditions? If so, how can this potential be realized to improve crop photosynthetic efficiency and productivity?

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