The Coordination of C_4 Photosynthesis and the CO_2-Concentrating Mechanism in Maize and *Miscanthus* × *giganteus* in Response to Transient Changes in Light Quality[1][W][OPEN]

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Unequal absorption of photons between photosystems I and II, and between bundle-sheath and mesophyll cells, are likely to affect the efficiency of the CO_2-concentrating mechanism in C_4 plants. Under steady-state conditions, it is expected that the biochemical distribution of energy (ATP and NADPH) and photosynthetic metabolite concentrations will adjust to maintain the efficiency of C_4 photosynthesis through the coordination of the C_3 (Calvin-Benson-Bassham) and C_4 (CO_2 pump) cycles. However, under transient conditions, changes in light quality will likely alter the coordination of the C_3 and C_4 cycles, influencing rates of CO_2 assimilation and decreasing the efficiency of the CO_2-concentrating mechanism. To test these hypotheses, we measured leaf gas exchange, leaf discrimination, chlorophyll fluorescence, electrochromatic shift, photosynthetic metabolite pools, and chloroplast movement in maize (*Zea mays*) and *Miscanthus* × *giganteus* following transitional changes in light quality. In both species, the rate of net CO_2 assimilation responded quickly to changes in light treatments, with lower rates of net CO_2 assimilation under blue light compared with red, green, and blue light, red light, and green light. Under steady state, the efficiency of CO_2-concentrating mechanisms was similar; however, transient changes affected the coordination of C_3 and C_4 cycles in *M. giganteus* but to a lesser extent in maize. The species differences in the ability to coordinate the activities of C_3 and C_4 cycles appear to be related to differences in the response of cyclic electron flux around photosystem I and potentially chloroplast rearrangement in response to changes in light quality.

The CO_2-concentrating mechanism in C_4 plants reduces the carbon lost through the photorespiratory pathway by limiting the oxygenation of ribulose-1,5-bisphosphate (RuBP) by the enzyme Rubisco (Brown and Smith, 1972; Sage, 1999). Through the compartmentalization of the C_4 cycle in the mesophyll cells and the C_3 cycle in the bundle-sheath cells (Hatch and Slack, 1966), C_4 plants suppress RuBP oxygenation by generating a high CO_2 partial pressure around Rubisco (Furbank and Hatch, 1987). To maintain high photosynthetic rates and efficient light energy utilization, the metabolic flux through the C_3 and C_4 cycles must be coordinated. However, coordination of the C_3 and C_4 cycles is likely disrupted due to rapid changes in environmental conditions, particularly changes in light availability (Evans et al., 2007; Tazoe et al., 2008).

Spatial and temporal variations in light environments, including both light quantity and quality, are expected to alter the coordination of the C_3 and C_4 cycles. For example, it has been suggested that the coordination of C_3 and C_4 cycles is altered by changes in light intensity (Henderson et al., 1992; Cousins et al., 2006; Tazoe et al., 2006, 2008; Kromdijk et al., 2008, 2010; Pengelly et al., 2010). However, more recent publications indicate that some of the proposed light sensitivity of the CO_2-concentrating mechanisms in C_4 plants can be attributed to oversimplifications of leaf models of carbon isotope discrimination ($\Delta^{13}C$), in particular, errors in estimates of bundle-sheath CO_2 partial pressure and omissions of respiratory fractionation (Ubierna et al., 2011, 2013). Alternatively, there is little

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information on the effects of light quality on the coordination of C_3 and C_4 cycle activities and the subsequent impact on net rate of CO_2 assimilation (A_{net}).

In C_3 plants, A_{net} is reduced under blue light compared with red or green light (Evans and Vogelmann, 2003; Loreto et al., 2009). This was attributed to differences in absorbance and wavelength-dependent differences in light penetration into leaves, where red and green light penetrate farther into leaves compared with blue light (Vogelmann and Evans, 2002; Evans and Vogelmann, 2003). Differences in light quality penetration into a leaf are likely to have profound impacts on C_4 photosynthesis, because the C_4 photosynthetic pathway requires the metabolic coordination of the mesophyll C_4 cycle and the bundle-sheath C_3 cycle. Indeed, Evans et al. (2007) observed a 50% reduction in the rate of CO_2 assimilation in *Flaveria bidentis* under blue light relative to white light at a light intensity of 350 μmol quanta m^{-2} s^{-1}. This was attributed to poor penetration of blue light into the bundle-sheath cells and subsequent insufficient production of ATP in the bundle-sheath cells to match the rates of mesophyll cell CO_2 pumping (Evans et al., 2007). Recently, Sun et al. (2012) observed similar low rates of steady-state CO_2 assimilation under blue light relative to red, green, and blue light (RGB), red light, and green light at a constant light intensity of 900 μmol quanta m^{-2} s^{-1}.

Because the light penetration into a leaf depends on light quality, with blue light penetrating the least, this potentially results in changes in the energy available for carboxylation reactions in the bundle-sheath (C_3 cycle) and mesophyll (C_4 cycle) cells. Changes in the balance of energy driving the C_3 and C_4 cycles can alter the efficiency of the CO_2-concentrating mechanisms, often represented by leakiness (ϕ), the fraction of CO_2 that is pumped into the bundle-sheath cells that subsequently leaks back out (Evans et al., 2007). Unfortunately, ϕ cannot be measured directly, but it can be estimated through the combined measured and modeled values of $\Delta^{13}C$ (Farquhar, 1983). Using measurements of $\Delta^{13}C$, it has been demonstrated that under steady-state conditions, changes in light quality do not affect ϕ (Sun et al., 2012); however, it remains unknown if ϕ is also constant during the transitions between different light qualities. In fact, sudden changes of light quality could temporally alter the coordination of the C_3 and C_4 cycles.

To understand the effects of light quality on C_4 photosynthesis and the coordination of the activities of C_3 and C_4 cycles, we measured transitional changes in leaf gas exchange and $\Delta^{13}C$ under RGB and broad-spectrum red, green, and blue light in the NADP-malic enzyme C_4 plants maize (*Zea mays*) and *Miscanthus* × *giganteus*. Leaf gas exchange and $\Delta^{13}C$ measurements were used to estimate ϕ using the complete model of C_4 leaf $\Delta^{13}C$ (Farquhar, 1983; Farquhar and Cernusak, 2012). Additionally, we measured photosynthetic metabolite pools, Rubisco activation state, chloroplast movement, and rates of linear versus cyclic electron flow during rapid transitions from red to blue light and blue to red light. We hypothesized that the limited penetration of blue light into the leaf would result in insufficient production of ATP in the bundle-sheath cells to match the rate of mesophyll cell CO_2 pumping. We predicted that rapid changes in light quality would affect the coordination of the C_3 and C_4 cycles and cause an increase in ϕ, but this would equilibrate as leaf metabolism reached a new steady-state condition.

RESULTS

Gas Exchange and Photosynthetic Discrimination

The A_{net} was significantly affected by changes in light treatment in both maize (Fig. 1A) and *M. giganteus* (Fig. 1B), with lower A_{net} under blue light compared with RGB, red, and green light at 900 μmol quanta m^{-2} s^{-1}. In maize, it took 20 to 30 min for A_{net} to stabilize when transitioned from either red or green light to blue light (Fig. 1A). However, in *M. giganteus*, A_{net} stabilized within minutes under these light transitions (Fig. 1B). Both stomatal conductance (g_s) and the ratio of intercellular to ambient CO_2 partial pressure (P_i/P_a) responded within minutes to changes in light quality in maize; however, g_s was lower and P_i/P_a was higher under blue light relative to other light treatments (Fig. 1, C and E). For *M. giganteus*, changing light treatment from RGB, red, and green to blue resulted in a gradual decrease in g_s, whereas switching light from blue to red, green, and RGB caused a gradual increase in g_s (Fig. 1D). Overall, the responses of g_s to variation in light treatment in *M. giganteus* were not as rapid as in maize, and P_i/P_a was higher under blue light relative to other light treatments in *M. giganteus* (Fig. 1F).

$\Delta^{13}C$ and ϕ in maize (Fig. 1, G and I) were less sensitive to changes in light treatments than in *M. giganteus* (Fig. 1, H and J). Values of $\Delta^{13}C$ and ϕ increased in *M. giganteus* during light transitions from blue to red, green, or RGB and decreased when switched from red or green to blue (Fig. 1, H and J). The $\Delta^{13}C$ in maize was linearly related to P_i/P_a under all light qualities, consistent with a constant ϕ (Fig. 2A); however, the response in *M. giganteus* indicated a shift in ϕ with light quality (Fig. 2B). In both species, $\Delta^{13}C$ and ϕ tended to converge to a constant value after 1 h of illumination in all light treatments (Figs. 1 and 2). Variation of $\Delta^{13}C$ in maize was mainly related to changes in P_i/P_a, whereas in *M. giganteus*, shifts of $\Delta^{13}C$ were associated with changes in both P_i/P_a and ϕ (Fig. 2).

The allocation of absorbed excitation energy between the C_3 and C_4 cycles (x) was modeled by solving for the value of x that minimizes the difference between the modeled (see Eq. 3) and measured values of $\Delta^{13}C$, assuming that bundle-sheath conductance did not change with light quality and was equal to values estimated under RGB. To account for the measured

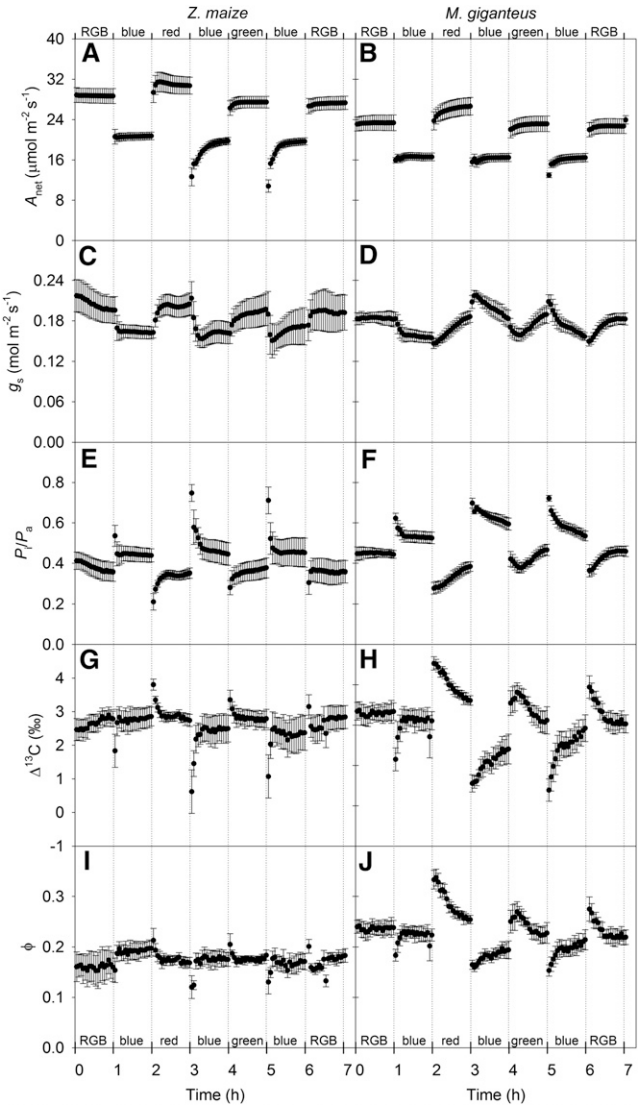

Figure 1. Transitional variation in A_{net} (μmol m^{-2} s^{-1}; A and B), g_s (mol m^{-2} s^{-1}; C and D), P_i/P_a (unitless; E and F), $\Delta^{13}C$ (‰; G and H), and ϕ (I and J) in maize and *M. giganteus* with changes in light treatment in the light sequence RGB, blue, red, blue, green, blue, and RGB. The light intensity was set to 900 μmol quanta m^{-2} s^{-1} for all light treatments. The CO_2 partial pressure in the leaf chamber was maintained at 38.4 Pa, leaf temperature was 25°C, and relative humidity was between 50% and 70% for all light treatments. Data are reported as means ± SE (n = 4).

3.5‰ increase in $\Delta^{13}C$ in *M. giganteus*, the x value had to increase 20%, with lower values under blue light compared with the other light treatments (Fig. 3). In maize, the required change in x needed to explain $\Delta^{13}C$ was small compared with that in *M. giganteus*. The modeled bundle-sheath CO_2 partial pressure (P_{bs}) was lower and the Rubisco oxygenation-carboxylation ratio (v_o/v_c) was higher under blue light compared with other light treatments in both maize and *M. giganteus* (Fig. 3, C–F).

Linear Electron Flux and Proton Motive Force

Measurements of A_{net}, g_s, and P_i/P_a made with the LI-COR 6400 coupled to the electrochromatic shift (ECS) system were similar to those made with the LI-COR 6400 coupled to the tunable diode laser absorption spectroscope (TDLAS; compare Fig. 1 and Supplemental Fig. S1). The efficiency of photosystem II (ϕ_{PSII}) was higher under red light compared with blue light for both maize and *M. giganteus* (Supplemental Fig. S2D). However, the total ECS was lower under blue light compared with red light, and this difference was larger in maize compared with *M. giganteus* (Supplemental Fig. S2E). Rates of proton flux across the thylakoid membrane (v_H^+) relative to rates of linear electron flux (LEF), or v_H^+/LEF, were lower under blue light compared with red light for both species. However, the shift in v_H^+/LEF was greater in maize compared with *M. giganteus* (Supplemental Fig. S2F). In both species, A_{net} was positively correlated with both ϕ_{PSII} (Fig. 4A) and v_H^+/LEF (Fig. 4B). In *M. giganteus*, ϕ was positively related to ϕ_{PSII}, and under a given light treatment, there was a negative relationship of ϕ and v_H^+/LEF (Fig. 4, C and D). In maize, ϕ did not change with shifts in ϕ_{PSII} and v_H^+/LEF in response to red or blue light (Fig. 4, C and D). It should be noted that v_H^+/LEF and ϕ were measured on different leaves in separate gas-exchange systems; however, for comparison, measurements of A_{net}, g_s and P_i/P_a from both setups are presented in Supplemental Figure S1.

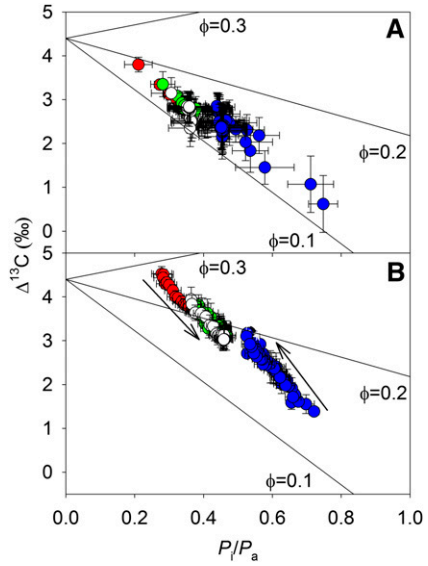

Figure 2. $\Delta^{13}C$ (‰) as a function of P_i/P_a (unitless) for RGB (white circles), red (red circles), green (green circles), and blue (blue circles) light in maize (A) and *M. giganteus* (B). Solid lines were modeled (Eq. 7) with ϕ of 0.1, 0.2, and 0.3 from bottom to top. Arrows indicate the convergence of ϕ to a constant value in *M. giganteus* after switching from one light species to another. Data are reported as means ± SE (n = 4).

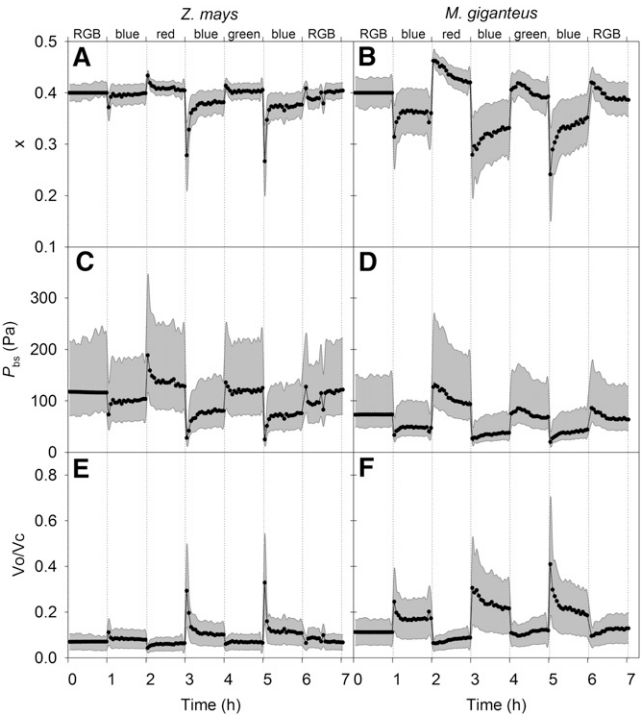

Figure 3. Modeled transitional variation in x (A and B), P_{bs} (Pa; C and D), and v_o/v_c (μmol m^{-2} s^{-1}; E and F) in maize and *M. giganteus* with changes in light quality in the order RGB, blue, red, blue, green, blue, and RGB at an irradiance of 900 μmol quanta m^{-2} s^{-1}. g_{bs} was estimated (0.0046 μmol m^{-2} s^{-1} Pa^{-1} for maize and 0.01 μmol m^{-2} s^{-1} Pa^{-1} for *M. giganteus*) using data obtained from the first 1 h of RGB measurements assuming x = 0.4. For the other light treatments, x was solved, assuming g_{bs} was constant, to minimize the least-square difference between modeled and measured Δ^{13}C. The gray areas show the sensitivity of the modeled parameters assuming different values of g_{bs} (between 0.0069 and 0.0023 μmol m^{-2} s^{-1} Pa^{-1} for maize and between 0.015 and 0.005 μmol m^{-2} s^{-1} Pa^{-1} for *M. giganteus*).

Pools of Photosynthetic Intermediates and Rubisco Activation State

There was more oxaloacetic acid (OAA) under blue light for both species (Supplemental Table S1); however, the change in concentration was slower in maize than in *M. giganteus* (Fig. 5A; Supplemental Table S2). Pyruvate was constant under red light, but there was a significant change in content with time after the transition to blue light for both species (Supplemental Table S2), which took 30 min to reach a steady state in *M. giganteus* and only 10 min in maize (Fig. 5C). It took 30 min for RuBP to reach steady state under red light in maize but only 10 min in *M. giganteus* (Fig. 5D). Under blue light transitions, RuBP increased more in *M. giganteus* than in maize (Fig. 5D). The amount of triose phosphate (TP) was significantly lower under blue light compared with red light, whereas pyruvate was higher under blue light compared with red light for both maize and *M. giganteus* (Fig. 5E; Supplemental Tables S1 and S2). Phospho*enol*pyruvate (PEP) and phosphoglycerate (PGA) were not significantly different between light treatments (Fig. 5F; Supplemental Table S2).

The ratios of C_3 metabolites (TP + RuBP + PGA) over C_4 metabolites (OAA + PEP + pyruvate) were significantly greater under red light compared with blue light treatment for both maize and *M. giganteus* (Fig. 6A; Supplemental Table S2). There was no statistically significant difference in PGA/PEP, but TP/PGA was lower under blue light compared with red light for both maize and *M. giganteus* (Fig. 6, B and D; Supplemental Table S2). The ratio of RuBP to PGA reached steady state within 10 min in *M. giganteus* but took 30 min in maize (Fig. 6C). After the transition to blue light, RuBP/PGA increased within 2 min for maize but took 10 min in *M. giganteus* (Fig. 6C). The activation state of Rubisco was constant across light treatments for both species (Supplemental Table S2).

Chloroplast Movement

Light quality-driven movement and positioning of chloroplast can be monitored through changes in leaf light transmittance (Inoue and Shibata, 1973; Trojan and Gabrys, 1996; DeBlasio et al., 2003). In leaves of maize and *M. giganteus*, chloroplast movement was monitored through changes in leaf transmittance (ΔT) during 1-h exposures to RGB, blue, and red light under similar conditions used for the gas exchange and online discrimination measurements. In *M. giganteus*, there was little ΔT over the 60-min time period regardless of light treatment (Fig. 7). However, in maize, there was a relatively large response of ΔT under all light treatments compared with *M. giganteus* (Fig. 7).

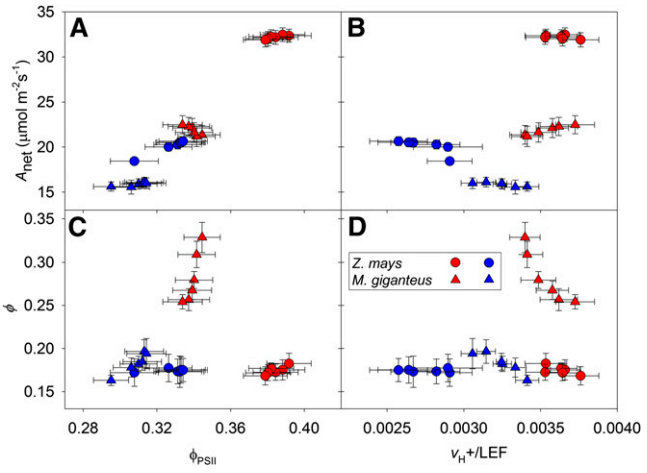

Figure 4. A_{net} (A and B) and ϕ (C and D) in response to variation in ϕ_{PSII} and v_H^+/LEF under red light (red symbols) and blue light (blue symbols) at 900 μmol quanta m^{-2} s^{-1}. Data are reported as means ± SE (n = 4–8).

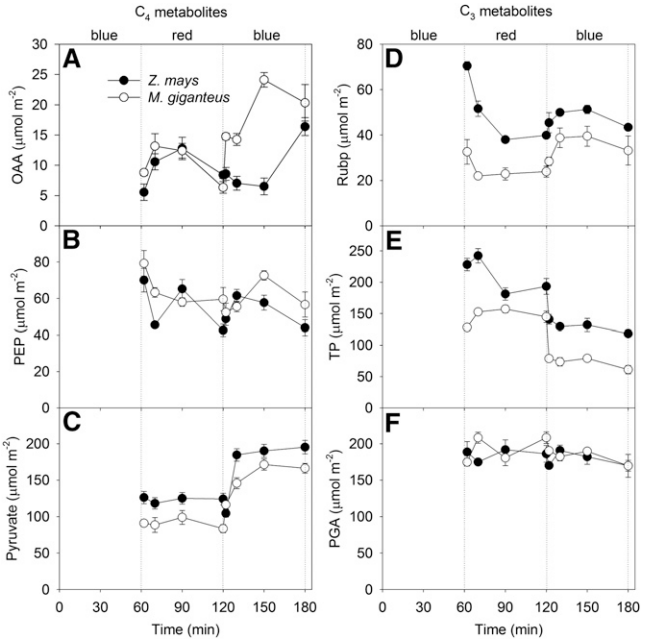

Figure 5. Transitional variation in pools of the C_4 metabolites OAA (μmol m^{-2}; A), pyruvate (μmol m^{-2}; B), and PEP (μmol m^{-2}; C) and the C_3 metabolites TP (μmol m^{-2}; D), RuBP (μmol m^{-2}; E), and PGA (μmol m^{-2}; F) in maize and *M. giganteus* when light was switched in the sequence blue, red, and blue at a light intensity of 900 μmol quanta m^{-2} s^{-1}. Data are reported as means ± SE (n = 4).

DISCUSSION

Light Quality and A_{net}

After a 1-h acclimation, the steady-state A_{net} was lower under blue light when compared with RGB, red light, and green light in both maize and *M. giganteus* (Fig. 1, A and B). The lower photosynthetic rate under blue light is consistent with previous findings for both C_3 (Evans and Vogelmann, 2003; Loreto et al., 2009) and C_4 (Evans et al., 2007; Sun et al., 2012) species. Low A_{net} under steady-state blue light has been discussed previously by Sun et al. (2012); however, in this study, there were changes in ϕ unrelated to changes in A_{net} that can provide insight into the mechanisms controlling the coordination of C_4 photosynthesis. For example, transitions from blue light to any other light quality lead to a relatively fast recovery of A_{net} in both species; however, ϕ increased sharply in *M. giganteus*, but there was little change in maize (Fig. 1). Additionally, in maize, A_{net} decreased significantly when shifted to blue light but without a change in ϕ (Fig. 1), suggesting that ϕ can be maintained regardless of changes in A_{net}. It has been demonstrated that A_{net} is typically determined by total available energy, whereas ϕ is determined by x (Siebke et al., 1997; von Caemmerer, 2000; Ubierna et al., 2011; Pengelly et al., 2012). Over time, the coordination of both cycles was achieved under all light qualities; however, the rate at which this balance was achieved differed between maize and *M. giganteus*.

Blue light reduction in rates of C_4 photosynthesis has been described before by Sun et al. (2012); therefore, this article focuses on the dynamic coordination between the C_3 and C_4 cycles during the transition from one light quality to another. In brief, the decrease in photosynthesis under blue light is the result of reduced energy availability to drive ATP and NADPH production. This is demonstrated by the measured changes in metabolite pools and the decrease in ϕ_{PSII} under blue light. First, the analysis of photosynthetic metabolite concentrations demonstrates lower ATP and NADPH availability under blue light when A_{net} is lower in both species. For example, pyruvate content under blue light was greater than under red light (Fig. 5C; Supplemental Table S2), which implies insufficient energy supply under blue light to convert pyruvate to PEP in mesophyll cells (Leegood and von Caemmerer, 1988, 1989; Sun et al., 2012). Moreover, TP content was lower under blue light relative to red light (Fig. 5E; Supplemental Table S2), whereas there were no differences in PGA content between red and blue light (Fig. 5F; Supplemental Table S2). These results suggest a lack of reducing equivalent for the reduction of PGA to TP.

There was also a strong positive relationship between A_{net} and ϕ_{PSII} (Fig. 4A) in response to light treatments, with lower ϕ_{PSII} under blue light (Supplemental Fig. S2). During C_4 photosynthesis, there is typically a linear dependence of ϕ_{PSII} and rates of CO_2 assimilation because the assimilation of CO_2 is the primary sink for NADPH generated by electron flux through PSII (Edwards and Baker, 1993). The lower ϕ_{PSII} under blue light observed in both species may result from (1) an imbalance in absorption between PSI and PSII within the mesophyll chloroplast, leading to an overreduction of the chloroplast electron transport chain (Evans, 1986);

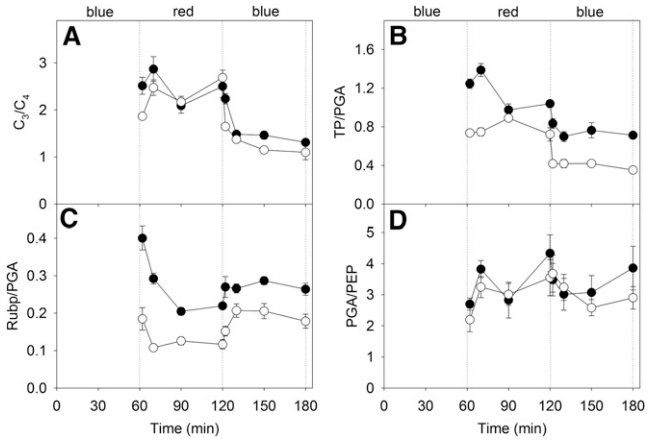

Figure 6. Transitional variation in the ratios of C_3 metabolites (TP + RuBP + PGA) over C_4 metabolites (OAA + PEP + pyruvate; A), TP over PGA (B), RuBP over PGA (C), and PEP over PGA (D) in maize (black circles) and *M. giganteus* (white circles) when light quality was switched in the sequence blue, red, and blue at a light intensity of 900 μmol quanta m^{-2} s^{-1}. Data are reported as means ± SE (n = 4).

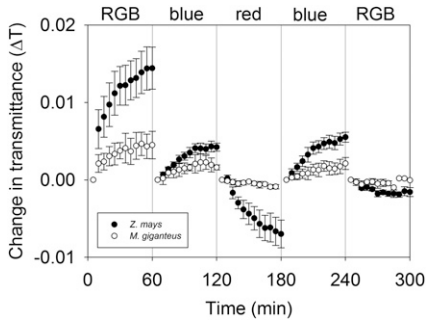

Figure 7. Transitional variation in ΔT in maize (black circles) and *M. giganteus* (white circles) when light treatment was switched in the sequence of RGB, blue, red, blue, and RGB. ΔT is defined as the difference between the initial averaged 5 min of transmittance of a given light quality and the transmittance at a given time point within the 60-min exposure. Data are reported as means ± SE (n = 4 for maize; n = 3 for *M. giganteus*).

(2) a change in the distribution of light energy absorption between the PSII-enriched mesophyll and the PSII-depleted bundle-sheath chloroplast; (3) an increase in nonphotochemical quenching; and/or (4) an overall decrease in the absorption of energy by the chlorophyll under blue light due to strong absorption of blue light by carotenoids and nonphotosynthetic structures within the leaf. As discussed below, some of these explanations will not only affect the total available energy but also x and, therefore, the overall efficiency of the CO_2-concentrating mechanism.

Coordination of the C_3 and C_4 Cycles in Response to Transient Changes in Light Quality

Measurements of $\Delta^{13}C$ indicated transitory changes in the coordination of the CO_2-concentrating mechanism in response to changes in light quality for both maize (Fig. 1I) and *M. giganteus* (Fig. 1J). This response lasted about 1 h in *M. giganteus* but only a few minutes in maize (Figs. 1 and 2). As discussed above, and in more detail below, these differences between species may be associated with the ability to coordinate the production of ATP and NADPH under changing light environments. Transition from any light treatment to blue light caused a reduction in $\Delta^{13}C$, while the reverse transition from blue light to any other light quality resulted in increased $\Delta^{13}C$ (Figs. 1 and 2).

A comparison of leaf-level models of $\Delta^{13}C$ and C_4 photosynthesis was used to investigate the variation of ϕ in response to rapid changes in light quality. This consisted of fitting measurements of $\Delta^{13}C$ and gas exchange into theoretical models of C_4 photosynthesis and leaf CO_2 discrimination (Ubierna et al., 2013) and solving for several parameters of interest. These included x, P_{bs}, and the rates of Rubisco oxygenation (v_o) versus carboxylation (v_c; Fig. 3). The C_4 photosynthesis model assumes steady-state conditions, which may be violated during the transition in light quality. However, there were no conditions where modeled parameters were outside physiologically feasible values. Additionally, this modeling assumed that the conductance of CO_2 between the bundle-sheath and mesophyll cells was constant. This may be an oversimplification; however, there is currently no evidence that bundle-sheath conductance (g_{bs}) changes in response to short-term manipulations in measurement conditions. Given these assumptions, these data illustrated that the transition from blue light to other light qualities resulted in a larger fraction of available energy allocated to the C_4 cycle, resulting in an increase in P_{bs}, a decrease in v_o/v_c, and an increase in ϕ (Fig. 3, A and B). The change in ϕ was larger in *M. giganteus* compared with maize. This suggests a faster coordination of the C_3 and C_4 cycles in maize compared with *M. giganteus*. Unfortunately, this modeling does not specifically identify the mechanism driving the change in energy allocation, but it does demonstrate that changes in light quality cause variations in the production of energy and reducing equivalents between the mesophyll and bundle-sheath cells.

Additionally, under blue light, there was a lower ratio of v_H^+/LEF (Fig. 4B). A lower v_H^+/LEF could be interpreted as lower activity of bundle-sheath cyclic electron flux; however, the ECS measurements integrate across the leaf cross section, and it is impossible to distinguish between the contributions of mesophyll and bundle-sheath cells. Nevertheless, it is reasonable that blue light would decrease cyclic electron flux preferentially in the bundle-sheath cells because of the limited penetration of blue light into the bundle-sheath cells (Evans and Vogelmann, 2003; Evans et al., 2007) and the insufficient blue light absorption by bundle-sheath PSI (Evans, 1986). This would limit the Calvin-Benson-Bassham cycle, because approximately 50% of the ATP requirement for carbon assimilation is thought to be provided by bundle-sheath cyclic electron flux (Kanai and Edwards, 1999). In *M. giganteus*, there was a positive relationship of ϕ and v_H^+/LEF, with higher ϕ and higher v_H^+/LEF under red light compared with blue light. However, over time after the initial change in light quality, there was a relatively large decrease in ϕ and only a slight increase in v_H^+/LEF for *M. giganteus*. Alternatively, in maize, there was no change in ϕ between light treatments, but v_H^+/LEF increased from blue to red light (Fig. 4D). This indicates that changes in v_H^+/LEF and corresponding changes in ATP/NADPH production in maize may help maintain a balance in metabolic flux between the C_3 and C_4 cycles and low ϕ. For example, in maize, increased cyclic electron flux under red light could drive additional phospho*enol*pyruvate carboxylase (PEPC) refixation of leaked CO_2 from the bundle-sheath cells, maintaining a constant $\Delta^{13}C$ and ϕ. In *M. giganteus*, there was only a small shift in v_H^+/LEF between blue and red light compared with maize, suggesting that change in cyclic electron flux in *M. giganteus* was not the only mechanism that ultimately balances the energy production and coordination between the C_3 and C_4 cycles. These findings in *M. giganteus* are

similar to those found in *F. bidentis*, where the ratio of PSI to PSII quantum yields did not change in plants with higher ϕ due to genetically reduced levels of Rubisco, suggesting that both linear and cyclic electron flux contribute to the production of ATP with increased ϕ (Siebke et al., 1997)

Chloroplast movement could also influence the metabolic and energy coordination between the C_3 and C_4 cycles. It has long been reported that blue light induces chloroplast movement to optimize photosynthetic activity and/or to prevent photodamage under strong light in both C_3 and C_4 species (Senn, 1908; Inoue and Shibata, 1974; Kagawa et al., 2001; Wada et al., 2003; Yamada et al., 2009; Luesse et al., 2010; Maai et al., 2011). In C_4 plants, blue light induced chloroplast movement in mesophyll but not in bundle-sheath cells (Yamada et al., 2009). This differential response could result in changes to the energy allocation between the C_3 (bundle-sheath) and C_4 (mesophyll) cycles, different rates of linear versus cyclic electron flux, and changes in the metabolite flux between both compartments, all of which could affect the coordination and efficiency of C_4 photosynthesis. There was more chloroplast movement, estimated as ΔT, in response to the transition in light quality in maize compared with *M. giganteus* (Fig. 7). This corresponded to the transitional variation in ϕ and x in response to light quality in *M. giganteus* but not in maize. Although the apparent chloroplast movement was relatively small, it does suggest that, in maize, it may help balance light energy distribution between the mesophyll and bundle-sheath cells, leading to a coordination of C_3 and C_4 cycle activities. In contrast, the lack of chloroplast movement in *M. giganteus* might be related to the longer time to coordinate the activities of C_3 and C_4 cycles in this species.

Although ϕ responded differently to light transitions in maize and *M. giganteus*, there were no significant differences in the rapid response of the contents and ratios of metabolites between species (Figs. 5 and 6; Supplemental Tables S1 and S2). However, as demonstrated previously by Sun et al. (2012), there were strong correlations between A_{net} and various metabolite concentrations (Figs. 5E and 6; Supplemental Tables S1 and S2). However, the lack of detectible transitional variation in photosynthetic metabolites may partially be attributed to the inability to measure metabolite fluxes between the mesophyll and bundle-sheath cells as well as an inability to determine mesophyll- or bundle-sheath-specific changes in metabolite concentrations.

Finally, the observed transitional variation in A_{net} and ϕ could be caused by light quality-associated differences in stomatal heterogeneity. Stomatal heterogeneity or patchiness has been observed in many species, particularly in response to environmental stress conditions (Terashima et al., 1988; Mott and Buckley, 1998). Stomatal heterogeneity could alter the estimation of $\Delta^{13}C$ and P_i/P_a by affecting the relationship between assimilation rate and stomatal conductance (Lloyd et al., 1992). However, Lloyd et al. (1992) demonstrated that the effects of stomatal heterogeneity on the estimation of $\Delta^{13}C$ are significant only under low light and low rates of stomatal conductance. We measured transitional variation in CO_2 assimilation rate and $\Delta^{13}C$ at a constant photon density of 900 μmol m^{-2} s^{-1} for all light treatments. Furthermore, the rates of stomatal conductance were relatively high under all light treatments (Fig. 1, C and D). Therefore, the effects of stomatal heterogeneity on the variation in A_{net} and $\Delta^{13}C$ are likely minimal in both species.

CONCLUSION

The in vivo coordination of the C_3 and C_4 cycles is complex and difficult to evaluate, requiring in many cases the interpretation of indirect evidence. We used several tools, including measurements of gas exchange, $\Delta^{13}C$, metabolite pools, ECS, and chloroplast movement, to characterize C_4 photosynthesis in response to changes in light quality. In both maize and *M. giganteus*, there was a lower efficiency of PSII under blue light, suggesting that the reduction in A_{net} under this condition was caused by insufficient production of NADPH through LEF. This was further supported by measurement of the pools of C_3 and C_4 metabolites. In maize, x was quickly optimized to minimize ϕ; however, it took almost 1 h for *M. giganteus* to achieve steady-state values of ϕ. Changes in linear versus cyclic electron flux in *M. giganteus* were slower to respond to light quality compared with those in maize. This may have led to an imbalance in the coordination of the C_3 and C_4 pathways. Additionally, the rapid rearrangement of chloroplasts under blue light in maize likely optimized light energy production between the mesophyll and bundle-sheath cells. Together, these responses in maize may have helped coordinate the C_4 photosynthetic pathway and potentially increased PEPC refixation of CO_2 leaked from the bundle-sheath cells. In *M. giganteus*, there was a quick change in ϕ_{PSII}, but cyclic electron flux and chloroplast movement were not as responsive to changes in light quality. This potentially resulted in an imbalance of energy, leading to the overpumping of CO_2 and increased ϕ. However, in both species, given enough time, the activities of C_3 and C_4 cycles appear to balance and maintain the efficiency of the C_4-concentrating mechanism.

MATERIALS AND METHODS

Plants

Maize (*Zea mays* var. Trucker's Favorite; Victory Seed Company) seeds and *Miscanthus* × *giganteus* rhizomes were planted in 6-L pots. The plants were grown in a greenhouse with day temperature of 25°C to 28°C, night temperature of 20°C to 25°C, and daylength of 14 h. Illumination in the greenhouse was a combination of sunlight and supplementary light provided by 400-W high-pressure sodium lamps. Plants were watered daily and fertilized weekly with Peters 20-20-20 (J.R. Peters) and a slow-release fertilizer (17-3-6 monopotassium phosphate). Four-week-old maize and 3-month-old *M. giganteus* were used for the measurements.

Gas Exchange and Online Leaf $\Delta^{13}C$

Leaf gas exchange and online $\Delta^{13}C$ were measured on the uppermost fully expanded maize and *M. giganteus* leaves using the LI-COR 6400xt gas-exchange analyzer with the opaque conifer chamber and the RGB light source (LI-COR 6400-18; LI-COR Biosciences) coupled to a TDLAS (TGA 100A; Campbell Scientific). The RGB light source generates a broad-spectrum red, green, and blue light with peak wavelengths of 635, 522, and 460 nm and bandwidths of 16, 35, and 24 nm, respectively. The $^{12}CO_2$ and $^{13}CO_2$ partial pressures in the LI-COR reference and sample cells were measured by the TDLAS concurrently with a CO_2-free tank and two standard tanks (Liquid Technology) with known $^{12}CO_2$ and $^{13}CO_2$ partial pressures. The TDLAS was sampled from each of the five sites at a flow rate of 150 mL min^{-1} and a frequency of 40 s per site. Only the last 10 s at each site was used for the calculation of $^{12}CO_2$ and $^{13}CO_2$ partial pressures. The partial pressures of $^{12}CO_2$ and $^{13}CO_2$ in the reference and sample lines were calibrated using a gain and offset calculated from the two calibration tanks (Bowling et al., 2003; Ubierna et al., 2011; Sun et al., 2012). The leaves were acclimated to 900 μmol m^{-2} s^{-1} RGB for 1 h in the opaque conifer chamber. The light treatment was then shifted in the order RGB, blue, red, blue, green, blue, and RGB, with an interval of 1 h per light treatment. The irradiance of 900 μmol m^{-2} s^{-1} was the maximum light intensity all light treatments could achieve with the LI-COR 6400-18 RGB light source. The CO_2 partial pressure in the leaf chamber was maintained at 38.4 Pa, a leaf temperature of 25°C, and a relative humidity between 50% and 70% for all gas-exchange measurements. The simultaneous gas exchange and $\Delta^{13}C$ measurements were conducted on four individual plants of maize and *M. giganteus*.

$\Delta^{13}C$ was calculated as (Evans et al., 1986):

$$\Delta^{13}C = \frac{1000 \times \xi(\delta_o - \delta_e)}{1000 + \delta_o - \xi(\delta_o - \delta_e)} \quad (1)$$

where δ_e and δ_o represent the isotopic composition of CO_2 entering and leaving the leaf chamber, respectively; ξ is calculated as:

$$\xi = P_e/(P_e - P_o) \quad (2)$$

where P_e and P_o are the partial pressures of CO_2 in the dry air entering and leaving the leaf chamber, respectively.

Leakiness

Following the recommendation of Ubierna et al. (2013), CO_2 ϕ was estimated by rearranging the equation proposed by Farquhar (1983) and Farquhar and Cernusak (2012):

$$\Phi = \frac{P_{bs} - P_i}{P_i} \times \frac{\Delta^{13}C(1-t)P_a - \overline{a}(P_a - P_i) - (1+t)P_i b_4}{(1+t)[b_3 P_{bs} - s(P_{bs} - P_i)] + \overline{a}(P_a - P_i) - P_a \Delta^{13}C(1-t)} \quad (3)$$

where P_a, P_i, and P_{bs} are CO_2 partial pressures in the atmosphere, the intercellular air spaces, and the bundle-sheath cells, respectively. The term $\overline{a}$ is the weighted fractionation across the boundary layer and stomata:

$$\overline{a} = \frac{a_b(P_a - P_L) + a_s(P_L - P_i)}{P_a - P_i} \quad (4)$$

where a_b (2.9‰) and a_s (4.4‰) are the fractionation across the boundary layer and stomata, respectively. P_L is CO_2 partial pressure at the leaf surface. The terms b_3 and b_4 are defined as (Farquhar, 1983):

$$b_3 = b_3' - \frac{e' R_d}{v_c} - \frac{f 0.5 v_o}{v_c} \quad (5)$$

$$b_4 = b_4' - \frac{e' R_m}{v_p} \quad (6)$$

where R_d is the nonphotorespiratory CO_2 release in the light (assumed to equal measured rates of dark respiration, 1.80 and 1.27 μmol m^{-2} s^{-1} for maize and *M. giganteus*, respectively); R_m is the mesophyll cell dark respiration rate, which is assumed to be half of the R_d (von Caemmerer, 2000); v_c and v_o are as already defined and v_p is the PEP carboxylation rate (for details on the modeling of v_c, v_o, v_p, and P_{bs}, see Ubierna et al. [2011]); b'_3 is fractionation by Rubisco, 30‰ (Roeske and O'Leary, 1984); b'_4 represents the net effect of CO_2 dissolution, hydration, and PEPC activity, which at 25°C has a value of −5.7‰ (Farquhar, 1983; Henderson et al., 1992); f is fractionation during photorespiration, 11.6‰ (Lanigan et al., 2008); and e' is fractionation associated with dark respiration, which is calculated as (Wingate et al., 2007):

$$e' = e + \delta^{13}C_{measurement} - \delta^{13}C_{growth} \quad (7)$$

where e is fractionation during decarboxylation, assumed to equal −6‰ (Ghashghaie et al., 2001; Sun et al., 2010); $\delta^{13}C_{measurement}$ (−31‰) is the carbon isotope signature of CO_2 used for the online discrimination measurement, which was measured by the TDLAS; and $\delta^{13}C_{growth}$ is the carbon isotope composition of CO_2 of the growth conditions, which is assumed to be −8‰.

The ternary effects term t is defined as (Farquhar and Cernusak, 2012):

$$t = \frac{\alpha_{ac} E}{2 g_{ac}} \quad (8)$$

where E is the transpiration rate, g_{ac} is the conductance to diffusion of air in CO_2, and $\alpha_{ac} (= 1 + \overline{a})$ is the fractionation factor for the isotopologs of CO_2 diffusing in air.

g_{bs} was estimated from the least-square difference between predicted and observed leaf discrimination using data obtained from the first 1-h RGB measurements (Kromdijk et al., 2010; Ubierna et al., 2011). This resulted in g_{bs} values of 0.0046 μmol m^{-2} s^{-1} Pa^{-1} for maize and 0.01 μmol m^{-2} s^{-1} Pa^{-1} for *M. giganteus*, which are within the range of values previously suggested for this parameter of 0.005 to 0.02 μmol m^{-2} s^{-1} Pa^{-1} (He and Edwards, 1996; von Caemmerer and Furbank, 2003).

Modeling Energy Partitioning between the C3 and C4 Cycles (x)

The light-limited C_4 photosynthesis model (von Caemmerer, 2000), which describes the relationships between total electron flux (J_t) and A_{net}, was used to model how x could explain the changes in $\Delta^{13}C$ observed in Figure 1. J_t can be partitioned between the C_4 cycle (J_m) and the C_3 cycle (J_s) by x, which is the portion of ATP required for J_m, and $1 - x$ is the requirement for J_s. The C_4 photosynthetic pathway has a theoretical minimum energetic cost of five ATPs, three for the C_3 cycle and two required to regenerate PEP from pyruvate in the mesophyll cells, so it is generally assumed that $x = 0.4$ (von Caemmerer, 2000). Therefore, during the first 1 h of RGB, x was assumed to be 0.4; however, for other light treatments, x was solved to minimize the least-square difference between modeled and measured $\Delta^{13}C$. Currently, there is no evidence that g_{bs} changes in response to variation in measurement conditions. Therefore, during this modeling, it was assumed that g_{bs} (0.0046 μmol m^{-2} s^{-1} Pa^{-1} for maize and 0.01 μmol m^{-2} s^{-1} Pa^{-1} for *M. giganteus*) and dark-type respiration (1.80 and 1.27 μmol m^{-2} s^{-1} for maize and *M. giganteus*, respectively) were constant for all light treatments and mesophyll conductance to CO_2 was infinite. Additionally, the modeled relationship between light, A_{net}, and J_t was used to calculate P_{bs} and v_o/v_c (Fig. 3), as described (Ubierna et al., 2011; Supplemental Equations S1). To demonstrate the sensitivity of x, P_{bs}, and v_o/v_c to g_{bs}, these parameters were modeled with g_{bs} of 0.0069 and 0.0023 μmol m^{-2} s^{-1} Pa^{-1} for maize and 0.015 and 0.005 μmol m^{-2} s^{-1} Pa^{-1} for *M. giganteus* (Fig. 3, shaded areas).

Chlorophyll *a* Fluorescence and ECS

Measurements were performed with a LI-COR 6400 gas-exchange analyzer coupled to a nonfocusing optics spectrophotometer/chlorophyll fluorometer (Avenson et al., 2004, 2005; Kiirats et al., 2010). In brief, 0.79 cm^2 of leaf was clamped into a custom-built leaf chamber that allowed simultaneous measurements of net CO_2 exchange, chlorophyll fluorescence, and leaf optical properties. The leaf chamber was maintained at 38.4 Pa of CO_2 and 900 μmol photon m^{-2} s^{-1}. Leaves were acclimated for 1 h under blue actinic light (460 nm), and then the actinic light was switched to red (637 nm) for 1 h and then to blue for 1 h. Measurements of chlorophyll *a* fluorescence and 520-nm absorbance in the dark were taken every 10 min per light treatment. The measuring and actinic light were the same wavelength for the measurements of chlorophyll *a* fluorescence.

The ϕ_{PSII} derived from fluorescence measurements (Genty et al., 1989) was used to estimate the LEF as $\phi_{PSII} \times Abs_{leaf} \times fraction_{PSII}$ (Donahue et al., 1997; Avenson et al., 2005), where Abs_{leaf} and $fraction_{PSII}$ are leaf absorbance and the fraction of light energy capture by PSII as compared with total photosystems, respectively. Leaf absorbance was determined from measurements of reflectance and transmittance as described previously by Sun et al. (2012), and the

$fraction_{PSII}$ was assumed to be 0.5. Absorbance changes at 520 nm from rapid light-to-dark transitions were used to estimate ECS. The total proton motive force was defined as the amplitude of ECS decay from steady state to a quasistable minimum after a 300-ms dark period (Avenson et al., 2004, 2005). The relative rate of v_{H^+} was estimated by ECS decay kinetics (Avenson et al., 2004, 2005; Livingston et al., 2010).

Rapid Freeze Clamping of Leaves and Photosynthetic Metabolite Measurements

Leaves similar in age and position to those used for the transitional gas exchange and $\Delta^{13}C$ measurements were rapidly freeze clamped in situ using a custom-built rapid kill system attached to a LI-COR 6400 gas-exchange analyzer (Badger et al., 1984; Hendrickson et al., 2008). Leaves were acclimated to either red or blue light for 1 h at a light intensity of 900 μmol quanta m^{-2} s^{-1}, a sample CO_2 partial pressure of 38.4 Pa, and a leaf temperature of 25°C. Leaves were then rapidly freeze clamped between two liquid nitrogen-cooled copper rods at 2, 10, 30, and 60 min after light was switched from red to blue or blue to red. The frozen leaf discs were stored in a −80°C deep freezer prior to extraction for photosynthetic metabolites and Rubisco activation state assays (Sun et al., 2012). Leaf discs (approximately 4.5 cm^2) were ground to a fine powder in liquid nitrogen in a precooled pestle and mortar and extracted with 1 M $HClO_4$ (Leegood and von Caemmerer, 1988). OAA, pyruvate, and PEP were measured consecutively in an enzymatic assay by monitoring changes in absorbance at 340 nm (Leegood and Furbank, 1984). TP, PGA, and RuBP were also assayed spectrophotometrically by coupled enzyme as described previously (He et al., 1997) using Rubisco purified from tobacco (*Nicotiana tabacum*).

Rubisco Activation State

Rubisco activity was determined by the incorporation of $^{14}CO_2$ into acid-stable products at 25°C (Salvucci and Anderson, 1987). The initial activity was determined by adding 20 μL of the leaf extract to the reaction mixture (100 mM Tricine-NaOH, pH 8.0, 10 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ [0.2 μCi μmol^{-1}], and 0.4 mM RuBP) and quenching the reaction after 30 s with 100 μL of acid (1 N HCl/4 N formic acid). Total activity was measured after incubating 20 μL of the same leaf extract in the reaction mixture for 3 min at 25°C minus RuBP to carbamylate all Rubisco catalytic sites. Subsequently, the reaction was initiated with RuBP and quenched after 30 s. Rubisco activation state was determined by the ratio of initial to total Rubisco activity.

Chloroplast Movement

Chloroplast movement was determined from the ΔT during exposure to different wavelengths of light (DeBlasio et al., 2003). Leaves were clamped to an integrating sphere (Labsphere) in a custom-built apparatus that included a multispectral light-emitting diode light source (Cree) that could be toggled between preset light qualities. Red, RGB, and blue light intensity was adjusted so that incident light had a similar intensity to that used during gas-exchange measurements. A photometer (LI-COR Biosciences) was attached to the integrating sphere, and light intensity was averaged over 5-min intervals using a data logger (Campbell Scientific). Leaves were exposed for 1 h each to the following light regime: RGB, blue, red, blue, and RGB. Reference traces were measured under the same regime without a leaf and used to calculate ΔT according to:

$$\Delta T = \frac{I_t}{I_r} - \frac{I_{t0}}{I_{r0}} \quad (9)$$

where I_t and I_r are the light intensities measured under a given light treatment and time point for the leaf and reference runs, respectively. I_{t0} and I_{r0} are the averaged light intensities from the leaf and reference runs measured during the initial 5 min of each light quality treatment. Therefore, ΔT is defined as the difference between the initial averaged 5 min of transmittance of a given light quality and the transmittance at a given time point within the 60-min exposure.

Statistical Analyses

Three-way ANOVA was conducted to assess the effects of species (maize and *M. giganteus*), time (2, 10, 30, and 60 min), and light treatment (red and blue) on the contents of OAA, pyruvate, PEP, RuBP, TP, and PGA, the ratios of C_3 to C_4 metabolites (TP to PGA, RuBP to PGA, and PGA to PEP), and Rubisco activation state. Statistical analyses were carried out using SAS version 9.0 (SAS Institute). Average values are reported as arithmetic means ± SE.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Comparison in gas exchange measurements between gas analyzers coupled to a tunable diode laser and an electrochromatic shift spectrophotometer.

Supplemental Figure S2. Transitional variation in gas exchange parameters, chlorophyll *a* fluorescence, and electrochromatic shift.

Supplemental Table S1. Metabolite profiles.

Supplemental Table S2. Statistical analysis.

Supplemental Equations S1. Calculation of total electron transport rate and CO_2 partial pressure inside the bundle-sheath cells.

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