






ORIGINAL RESEARCH

C₄ monocots and C₄ dicots exhibit rapid photosynthetic induction response in contrast to C₃ plants

Keiichiro Tanigawa¹  | Qu Yuchen¹ | Naoya Katsuhama¹  |
 Kazuma Sakoda^{1,2}  | Yu Wakabayashi¹ | Yu Tanaka³ | Rowan Sage⁴ |
 Tracy Lawson⁵  | Wataru Yamori¹ 

¹Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

²Space Environment and Energy Laboratories, Nippon Telegraph and Telephone Corporation, Tokyo, Japan

³Graduate School of Environmental, Life, Natural Science and Technology, Okayama University, Okayama, Japan

⁴Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON, Canada

⁵School of Life Sciences, University of Essex, Colchester, UK

Correspondence

Wataru Yamori, Institute for Sustainable Agroecosystem Services, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 188-0002, Japan.
 Email: yamori@g.ecc.u-tokyo.ac.jp

Funding information

Japan Society for the Promotion of Science, Grant/Award Numbers: 18KK0170, 20H05687, 21H02171, 24A301, 24H02277

Edited by F. Busch

Abstract

Considering the prevalence of ever-changing conditions in the natural world, investigation of photosynthetic responses in C₄ plants under fluctuating light is needed. Here, we studied the effect of dynamic illumination on photosynthesis in totally 10 C₃, C₃-C₄ intermediate, C₄-like and C₄ dicots and monocots at CO₂ concentrations of 400 and 800 μmol mol⁻¹. C₄ and C₄-like plants had faster photosynthetic induction and light-induced stomatal dynamics than C₃ plants at 400 μmol mol⁻¹, but not at 800 μmol mol⁻¹ CO₂, at which the CO₂ supply rarely limits photosynthesis. C₄ and C₄-like plants had a higher water use efficiency than C₃ plants at both CO₂ concentrations. There were positive correlations between photosynthetic induction and light-induced stomatal response, together with CO₂ compensation point, which was a parameter of the CO₂-concentrating mechanism of C₄ photosynthesis. These results clearly show that C₄ photosynthesis in both monocots and dicots adapts to fluctuating light conditions more efficiently than C₃ photosynthesis. The rapid photosynthetic induction response in C₄ plants can be attributed to the rapid stomatal dynamics, the CO₂-concentrating mechanism or both.

1 | INTRODUCTION

In their natural habitat, plants are exposed to a range of light intensities, including morning induction of photosynthesis at dawn, and frequently exposed to fluctuating light intensities throughout the diurnal period due to factors such as variable cloud cover, self-shading within the canopy, and leaf movement. Plants in different growing habitats and locations will experience different dynamic patterns of light depending on whether they are understory herbaceous species or monoculture crops in an open field. Plant carbon gain and biomass

production are generally lower under fluctuating light compared to constant light due to changes in stomatal conductance, enzyme kinetics and metabolite pool sizes (Percy, 1990). To assess the contribution of stomatal limitation to carbon gain, several studies have examined the induction of photosynthesis from darkness to saturating light (Kaiser et al., 2016; Papanatsiou et al., 2019; Shimadzu et al., 2019; Kimura et al., 2020; Sakoda et al. 2021). Simulation analyses have revealed that the potential loss in daily carbon gain from slow photosynthetic induction can exceed 20% in soybean (*Glycine max* (L.) Merr.) (Tanaka et al., 2019) and wheat (*Triticum aestivum* L.)

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(Taylor and Long, 2017) under conditions mimicking natural light fluctuations found in field settings.

In recent years, there has been a surge in research dedicated to investigating photosynthetic induction, driven by the growing recognition of its significance to crop yields under fluctuating light conditions (e.g., Kromdijk et al., 2016; Murchie et al., 2018; Papanatsiou et al., 2019; Yamori et al., 2020). Photosynthetic induction is mainly limited by activation of Calvin Cycle enzymes in the initial stage, and stomatal opening in later stage (Percy et al., 1996; Lawson et al., 2010; 2012). Biochemical control related with Calvin Cycle typically take 3–10 min to fully response to changing light conditions, with greater periods reported for understory species (Percy et al., 1996) compared to crops (e.g. Acevedo-Siaca et al., 2020; Long et al., 2022). Then, it is followed by diffusional constraints caused by low stomatal conductance (g_s) which last for longer period (Lawson & Blatt., 2014; Lawson & Vialet-Chabrand 2019). The rate of photosynthetic induction greatly increases at higher CO_2 partial pressure, due to faster Rubisco activation rates and reduced diffusional limitations (Kaiser et al., 2016; Shimadzu et al., 2019), however, other environmental factors, such as temperature and VPD also have an impact (e.g. high temperature resulted in faster induction, whilst high VPD slowed induction rates). At ambient CO_2 , maximising Rubisco activation rates could improve photosynthesis by 6–8% (Kaiser et al., 2016), whilst removing diffusional constraints could improve photosynthesis by 10% (McAusland et al., 2016). There are many contrasting reports regarding the extent and duration of biochemical vs stomatal limitation on photosynthetic induction, with some papers suggesting biochemical limitations being more important (Yamori et al., 2012; Carmo-Silva et al., 2013; Kaiser et al., 2016) whilst others show greater diffusional limitations (McAusland et al., 2016; Shimadzu et al., 2019; Kimura et al., 2020; Yamori et al., 2020). Both could be true in some extreme cases, for example, biochemistry accounted for 100% of the initial limitation in rice, however this dropped rapidly to less than 20% within three minutes, with stomatal limitation in selected species constraining photosynthesis by 1/3 over the subsequent 10 min (Acevedo-Siaca et al., 2020).

Recent investigations involving model plants, mutants or transgenic plants have revealed that the rate of stomatal opening can limit photosynthetic induction in C_3 plants (McAusland et al., 2016; Papanatsiou et al., 2019; Shimadzu et al., 2019; Kimura et al., 2020; Yamori et al., 2020), however crops vary greatly in the extent to which this is the case (Acevedo-Siaca et al., 2020, De Souza & Long., 2018; McAusland et al., 2016). Additionally, whilst ‘forgone’ CO_2 assimilation will increase if stomata open too slowly during induction, there are negative implications for water use efficiency if stomata open too rapidly (Long et al., 2022; Yoshiyama et al., 2024) and there are differences in these speeds of stomatal response between C_3 and C_4 plants (McAusland et al., 2016; Israel et al., 2022).

Despite their importance as crops, our understanding of the photosynthetic response in C_4 plants under fluctuating light, including photosynthetic induction, remains limited. C_4 plants are equipped with a CO_2 -concentrating mechanism that mitigates photorespiration (the wasteful process of Rubisco fixing O_2 instead of CO_2) and enhances CO_2 assimilation (Leegood, 2002; Keely and Rundel, 2003).

It is believed that C_4 plants evolved from C_3 plants through various C_3 – C_4 intermediate stages, wherein a photorespiration-dependent CO_2 -concentrating system, referred to as C_2 photosynthesis, operates (Sage et al., 2014). In the C_4 photosynthesis pathway, carbon dioxide (CO_2) acquired by mesophyll cells is converted into a four-carbon (C_4) compound by the enzyme phosphoenolpyruvate carboxylase, which has a high affinity for CO_2 . Subsequently, the C_4 compound is translocated to the chloroplasts of bundle sheath cells, where CO_2 is released, elevating the CO_2 concentration at the site of fixation and enhancing the carboxylase activity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), the rate-limiting enzyme in photosynthesis (Leegood, 2002; Keely and Rundel, 2003; Yamori et al., 2014). Under the prevailing atmospheric CO_2 concentration (i.e., $\sim 420 \mu\text{mol mol}^{-1}$), C_4 plants are already saturated with CO_2 and have higher CO_2 assimilation rates, greater water-use efficiency, and better nitrogen-use efficiency than C_3 plants (Sage and Percy, 2000).

The metabolic and structural requirements of the C_4 pathway may prevent C_4 plants from becoming as adaptive to environments as C_3 plants. It is a common assumption that the relative scarcity of C_4 plants in shaded habitats and forests stems from their less advanced adaptation to low-light conditions (Sage and McKown, 2006; Kubásek et al., 2013). Indeed, reports indicate a notable rarity of C_4 plants in forest understoreys (Smith and Martin 1987a, b; Horton and Neufeld 1998). Several constraints are evident in C_4 photosynthesis compared with C_3 plants within understorey conditions: (1) C_4 plants have a lower quantum yield for CO_2 uptake under low light, largely due to the additional energy consumption of the CO_2 -concentrating mechanism (Ehleringer and Björkman, 1977; Krall and Percy, 1993). (2) The CO_2 -concentrating mechanism appears to be less effective under low light owing to higher CO_2 leakage (Tazoe et al., 2008; Kromdijk et al., 2010). (3) The coordination of C_3 and C_4 cycles may be disrupted under fluctuating light, owing to the partially independent activation of C_3 and C_4 carboxylation enzymes (Smith et al., 1998).

Recent studies have explored the differences in photosynthetic capacity represented by CO_2 assimilation rate (A) and g_s under fluctuating light conditions between C_3 and C_4 plants, but their findings remain inconclusive. Some studies reported that C_4 plants have lower light-use efficiency than C_3 plants under fluctuating light, drawing this conclusion from observations of eight C_3 and six C_4 plants representing a phylogenetically diverse range of dicots and monocots (Li et al., 2021) or observations of three pairs of phylogenetically controlled C_3 and C_4 plants (Arce Cubas et al., 2023). In contrast, other studies suggested that C_4 plants assimilate more carbon than C_3 plants under fluctuating light (McAusland et al., 2016; Lee et al. 2021; Ozeki et al., 2022; Suwannarut et al., 2023). Consequently, it is important to develop a comprehensive understanding of whether or not C_4 plants photosynthesize perform less efficiently than C_3 plants under the fluctuating light conditions commonly encountered in natural settings.

Specifically, during dark light transition, the coordination of C_4 and C_3 cycles may be impacted (Slattery et al., 2018), leading to a reduction in C-fixation efficiency in C_4 plants during photosynthetic induction (Arce Cubas et al., 2023). Synchronisation relies on C_4 acid transport into bundle sheath cells building up a gradient, however pool size is dependent on light intensity and can result in suboptimal concentrations of CO_2 near Rubisco, during low to high light

transitions (Slattery et al., 2018), which could increase photorespiration during the period of photoinduction (Kromdijk et al., 2010; Medeiros et al., 2022). Whilst mechanisms behind slower activation of CO₂ assimilation in C₄ species varies, incomplete suppression of photorespiration was the main contributor in C₄ *Flaveria bidentis* (Arce Cubas et al., 2023). Several studies have also shown the involvement of photorespiratory processes in modifying *g_s* (Eisenhut et al., 2017; Fluegel et al., 2017; Timm et al., 2019), impacting on stomatal kinetics and thus potentially contribute to diffusional constraints.

Furthermore, global concentrations of atmospheric CO₂, a crucial factor influencing photosynthesis, have been increasing in recent years and are projected to continue rising (IPCC, 2022). As the leaves of C₄ crops are already saturated by existing atmospheric CO₂ concentration, smaller guard cells resulting in faster stomata in these species could confer a benefit in terms of water use efficiency, as *g_s* could be reduced without impacting on CO₂ uptake (Long and Spence 2013; Pignon and Long, 2020). To the best of our knowledge, no studies have analysed the photosynthetic induction response under varying CO₂ concentrations in both C₃ and C₄ plants. Hence, it is essential to understand how C₃ and C₄ plants respond to fluctuating light conditions under the current and expected higher atmospheric CO₂ concentrations.

Here, we analysed the CO₂ response of steady-state photosynthesis among eight dicots—species of genus *Flaveria*, which contains closely related C₃, C₃-C₄ intermediate, C₄-like and C₄ plants which are useful for studying the evolution of C₄ plants; and two monocots—*Oryza sativa* (C₃) cultivar and *Echinochloa oryzicola* (C₄: NADP-ME type). We then characterized the photosynthetic response to changing light intensity at CO₂ concentrations of 400 and 800 μmol mol⁻¹. These approaches enabled us to investigate the effects of phylogenetic history and photosynthetic processes independently, providing a more comprehensive understanding of the factors determining plant responses to the changing environments. They also help to identify potential evolutionary steps in the development of plants with distinct photosynthetic pathways.

2 | MATERIALS AND METHODS

2.1 | Plant materials and cultivation

We cultivated the monocots *Echinochloa oryzicola* (C₄: NADP-ME type) and *Oryza sativa* 'Koshihikari' (C₃), and the dicots *Flaveria pringlei* (C₃), *F. robusta* (C₃), *F. floridana* (C₃-C₄), *F. ramosissima* (C₃-C₄), *F. brownii* (C₄-like), *F. palmeri* (C₄-like), *F. bidentis* (C₄) and *F. trinervia* (C₄). The specific phylogeny of these *Flaveria* species was described by McKown et al. (2005). The *Flaveria* species were cultivated according to Taniguchi et al. (2021). All plants were grown in 5-L pots containing red granular Akadama soil and 4.0 g of a slow-release fertilizer. Plants were grown from April to August in a shaded greenhouse at the University of Tokyo (35°43'N, 139°32'E). The average air temperature and relative humidity in the glasshouse during the growing period were 30.6°C and 62.3%. The maximum light intensity in the greenhouse was 1500 μmol m⁻² s⁻¹. The plants were watered regularly, and all experiments used the uppermost, fully expanded leaves of 50- to 80-day-old plants.

2.2 | Simultaneous measurements of gas exchange and chlorophyll fluorescence

Gas exchange was measured in fully expanded young leaves with a portable gas exchange system (LI-6400XT, Li-Cor, Lincoln, NE, USA), according to Qu et al. (2021) and Yoshiyama et al. (2024). CO₂ assimilation rate (A) was measured under a light intensity of 1500 μmol m⁻² s⁻¹. The CO₂ concentration in the chamber was first held at 400 μmol mol⁻¹ under high light until A reached steady state, then it was decreased to 40 μmol mol⁻¹ and raised in a stepwise fashion to 80, 120, 160, 200, 400, 800, 1200 and 1500 μmol mol⁻¹ with at least 3 min of acclimatization at each stage.

Chlorophyll (Chl)-a fluorescence was determined simultaneously with an integrated fluorescence chamber head (LI-6400, LI-6400-40 leaf chamber fluorometer). First, leaves of plants that had been held in darkness overnight were treated with a saturating pulse to obtain maximum fluorescence. After measurement of the quantum yield of photosystem II (Φ_{PSII}) at various measurement conditions, we calculated the electron transport rate (ETR) through photosystem II as:

$$ETR = 0.5 \times I_{\text{abs}} \times \Phi_{\text{PSII}} \quad (\text{Equation 1})$$

where 0.5 is the fraction of absorbed light allocated to photosystems, and *I_{abs}* is the absorbed irradiance, taken as 0.84 of incident irradiance. Although it is challenging to measure the photosynthetic ETRs of mesophyll and bundle sheath chloroplasts individually within an intact C₄ leaf, the Chl fluorescence method offers a valuable tool for doing so indirectly (Genty et al., 1989; Krall et al., 1991; Oberhuber et al., 1993; Kiirats et al., 2010). Hence, we measured Chl fluorescence in intact leaves, assuming that the results represent composite signals originating from both types of chloroplasts within an intact leaf, as PSII accumulates in both mesophyll and bundle sheath chloroplasts of *F. bidentis* (Ketchner and Sayre, 1992; Meister et al., 1996; Ishikawa et al., 2016).

For the investigation of photosynthetic induction, plants were selected at random and measured from 07:00 to 15:00 to avoid confounding species with time of day and to minimize any diurnal influences. The day before the measurements were taken, plants were kept in the dark room at an air temperature of 25°C, relative humidity of 65% and ambient CO₂ concentration. A leaf was then placed into the measuring chamber whilst maintain the dark conditions, with an air temperature of 25°C, relative humidity of 65% and 400 or 800 μmol CO₂ mol⁻¹. During an initial 15-min period, a leaf was kept in dark conditions for acclimatization in the gas exchange system, and then were subsequently illuminated with a PPFD of 1500 μmol m⁻² s⁻¹ for 75 min. Although such an extreme dark-light transition rarely occurs in nature, we chose it to maximize the effects on the photosynthetic induction response (Allen and Percy, 2000; Urban et al., 2007; Kaiser et al., 2017; Guo et al., 2016; Zhang et al., 2018; Yoshiyama et al., 2024). A, stomatal conductance (*g_s*), intercellular CO₂ concentration (*C_i*) and Chl fluorescence were recorded at the same time, because this photosynthetic induction includes both biochemical and stomatal responses. Measuring Chl fluorescence required more than one minute intervals between measuring to ensure that repeated saturating pulses

do not become actinic. Therefore, measurements were recorded every 90 seconds in this study. To evaluate the rate of induction of A , g_s and ETR, we calculated the relative values of these parameters (X) as (Sakoda et al., 2020):

$$X = (X_t - X_{\min}) / (X_{\max} - X_{\min}) \quad (\text{Equation 2})$$

where X_t is the value at a given time under a PPFD of $1500 \mu\text{mol}\cdot\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, X_{\min} is the steady-state value during initial darkness, and X_{\max} is the maximum value under a PPFD of $1500 \mu\text{mol}\cdot\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

We evaluated the time when each parameter reached 50% (t_{50}) and 90% (t_{90}) of maximum after each step increase in light. Before evaluating the t_{50} and the t_{90} , the plot sequences of A , g_s and ETR were fitted to a Boltzmann sigmoidal function according to Sakoda et al. (2021). The fitness of these fitting curves was evaluated by QQ-plot (Figure S1). After the confirmation of these curve fittings, we calculated the t_{50} and the t_{90} using these regression curve to decide accurate values of them.

2.3 | Statistical analysis

Differences between two groups were tested using Student's t -test, and differences among three or more groups were analysed by ANOVA with Tukey-Kramer test according to Sakoda et al. (2021). Pearson's correlation coefficient (r) was calculated, and the significance of relationships was tested by two-sided t -tests ($P < 0.05$). All statistical analyses were conducted using R versi 3.6.1 software (R Foundation for Statistical Computing, Vienna, Austria).

3 | RESULTS

3.1 | CO_2 response of photosynthesis

We compared steady-state A plotted against C_i (A - C_i curve) among species (Figure 1). Among the dicots, A was higher in C_4 and C_4 -like plants than in C_3 and C_3 - C_4 plants at $C_i < 500 \mu\text{mol mol}^{-1}$, but was similar among species at $C_i > 500 \mu\text{mol mol}^{-1}$, except in *F. palmeri* (Figure 1A). Among the monocots, A was higher in C_4 plants than in C_3 plants at $C_i < 500 \mu\text{mol mol}^{-1}$, but was similar between species at $C_i > 500 \mu\text{mol mol}^{-1}$ (Figure 1B).

3.2 | Photosynthetic induction under ambient and elevated CO_2

The species showed significant variations in the induction response of all parameters at 400 and 800 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ (Figures 2, 3). At 400 $\mu\text{mol CO}_2 \text{ mol}^{-1}$, C_4 and C_4 -like dicots had higher A and intrinsic water use efficiency (iWUE = A/g_s) and lower g_s and C_i than C_3 and C_3 - C_4 dicots (Figure 2A-F); similarly, the C_4 monocot had higher A and iWUE and lower g_s and C_i than the C_3 monocots (Figure 2J-O). At 800 $\mu\text{mol CO}_2$

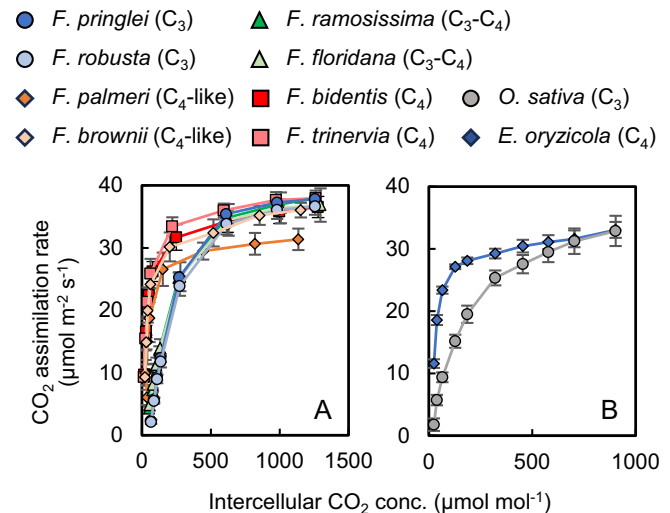


FIGURE 1 Responses of CO_2 assimilation rate in (A) C_3 , C_3 - C_4 , C_4 -like and C_4 dicot species and (B) C_3 and C_4 monocot species at a PPFD of $1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and an air temperature of 25°C . Dicots: C_3 (*Flaveria pringlei*, *F. robusta*), C_3 - C_4 (*F. ramosissima*, *F. floridana*), C_4 -like (*F. palmeri*, *F. brownii*) and C_4 (*F. bidentis*, *F. trinervia*). Monocots: C_3 (*Oryza sativa*) and C_4 (*Echinochloa oryzicola*). Data are means \pm SE ($n = 4$ -5).

mol^{-1} , C_4 and C_4 -like dicots had higher iWUE and lower g_s and C_i than C_3 and C_3 - C_4 dicots (Figure 3A-F); similarly, the C_4 monocot had higher iWUE and lower g_s and C_i than the C_3 monocots (Figure 3J-O). The C_3 - C_4 *F. ramosissima* had the lowest A , g_s and ETR but the highest C_i of all species under both CO_2 concentrations (Figure 3A-D).

We calculated t_{50} and t_{90} for the induction responses and found significant differences in t_{50} or t_{90} for A and g_s at 400 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ among the dicot groups (Figure 4A, B). t_{90} (but not t_{50}) for A was significantly lower in C_4 and C_4 -like plants than in C_3 and C_3 - C_4 plants (Figure 4A). t_{50} and t_{90} for g_s were significantly lower in C_4 plants than in C_3 and C_3 - C_4 plants (Figure 4B). There was no significant variation in t_{50} or t_{90} for ETR among species (Figure 4C), although t_{90} for ETR tended to be lower in C_4 plants than in the other groups (Figure 4C). No significant variations in t_{50} or t_{90} for A or g_s between C_4 and C_4 -like plants or between C_3 and C_3 - C_4 plants was observed (Figure 4A). Among the monocot group, we also found similar differences. t_{90} for A and g_s were significantly lower in C_4 plants than in C_3 plants (Figure 4D, E). There was no significant variation in t_{90} of ETR or in t_{50} for A , g_s , or ETR among species (Figure 4D-F).

At 800 $\mu\text{mol CO}_2 \text{ mol}^{-1}$, there were no significant variations in t_{50} or t_{90} for A among the dicot group (Figure 5A). However, a small difference in t_{50} and t_{90} for g_s and ETR was observed (Figure 5B, C). t_{50} and t_{90} for g_s were lowest in C_4 plants, intermediate in C_3 and C_4 -like plants, and highest in C_3 - C_4 plants (Figure 5B). t_{50} and t_{90} for ETR were lowest in C_3 - C_4 plants, intermediate in C_4 -like plants, and highest in C_3 and C_4 plants (Figure 5C). Among the monocot group, we also found similar relationships in t_{50} or t_{90} for A and g_s , although there were significant differences in t_{50} and t_{90} for ETR between dicots and monocots (Figure 5 D-F). There was no significant variation in t_{50} or t_{90} for A and ETR between C_3 and C_4 plants (Figure 5 D, F), whilst t_{50} and t_{90} for g_s was lower in C_4 plants than in C_3 plants (Figure 5 E).

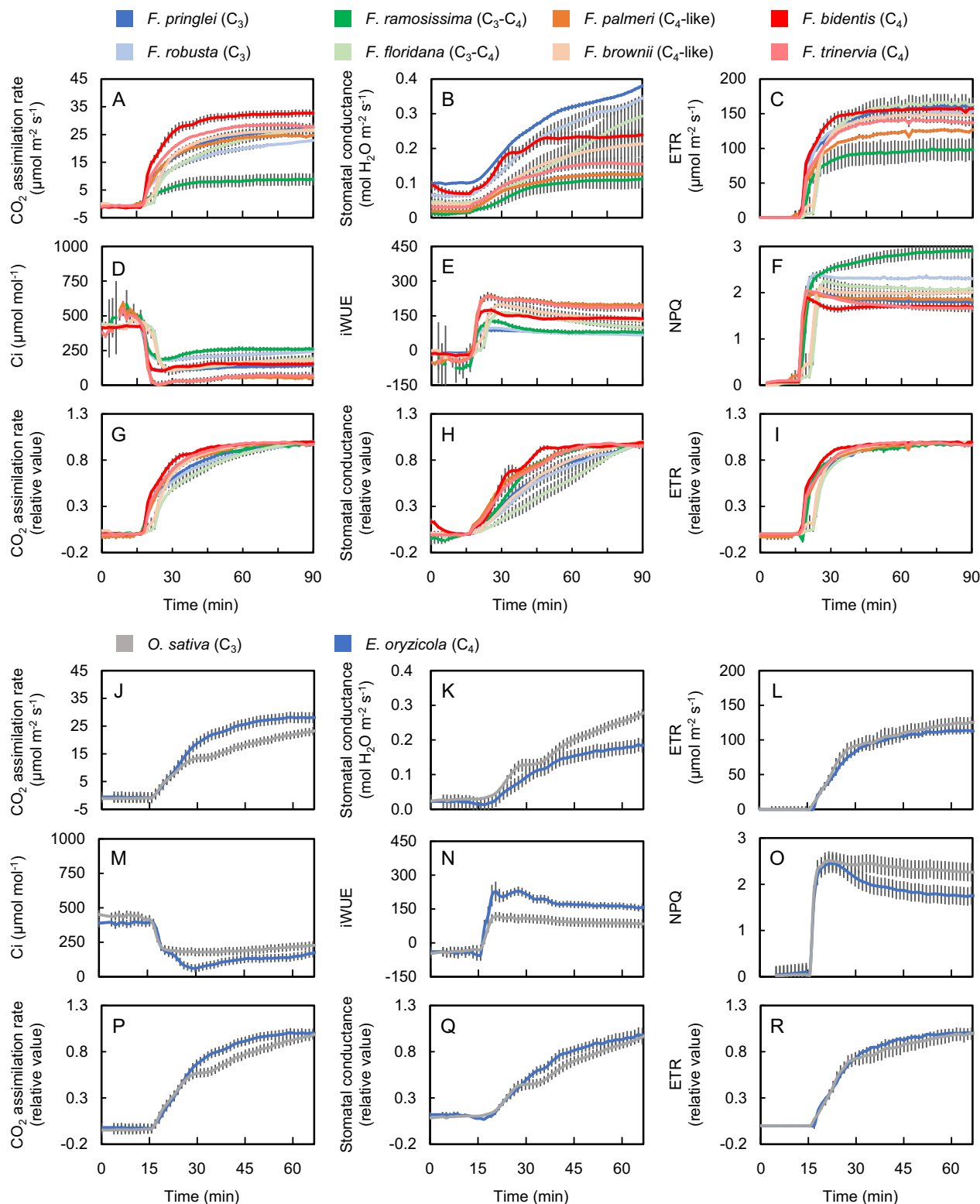


FIGURE 2 Induction responses of gas exchange and chlorophyll fluorescence parameters at $400 \mu\text{mol CO}_2 \text{ mol}^{-1}$ in (A-I) C₃, C₃-C₄, C₄-like and C₄ dicot species and in (J-R) C₃ and C₄ monocot species after step increases in light from darkness to PPFD = $1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ over 75 min. (A, J) CO₂ assimilation rate (A), (B, K) stomatal conductance (g_s), (C, L) electron transport rate (ETR), (D, M) intercellular CO₂ concentration (C_i), (E, N) intrinsic water use efficiency (iWUE), (F, O) non photochemical quenching (NPQ). Equation 1 was used to estimate relative values of (G, P) A, (H, Q) g_s , and (I, R) ETR. Data are means \pm SE ($n = 4-5$).

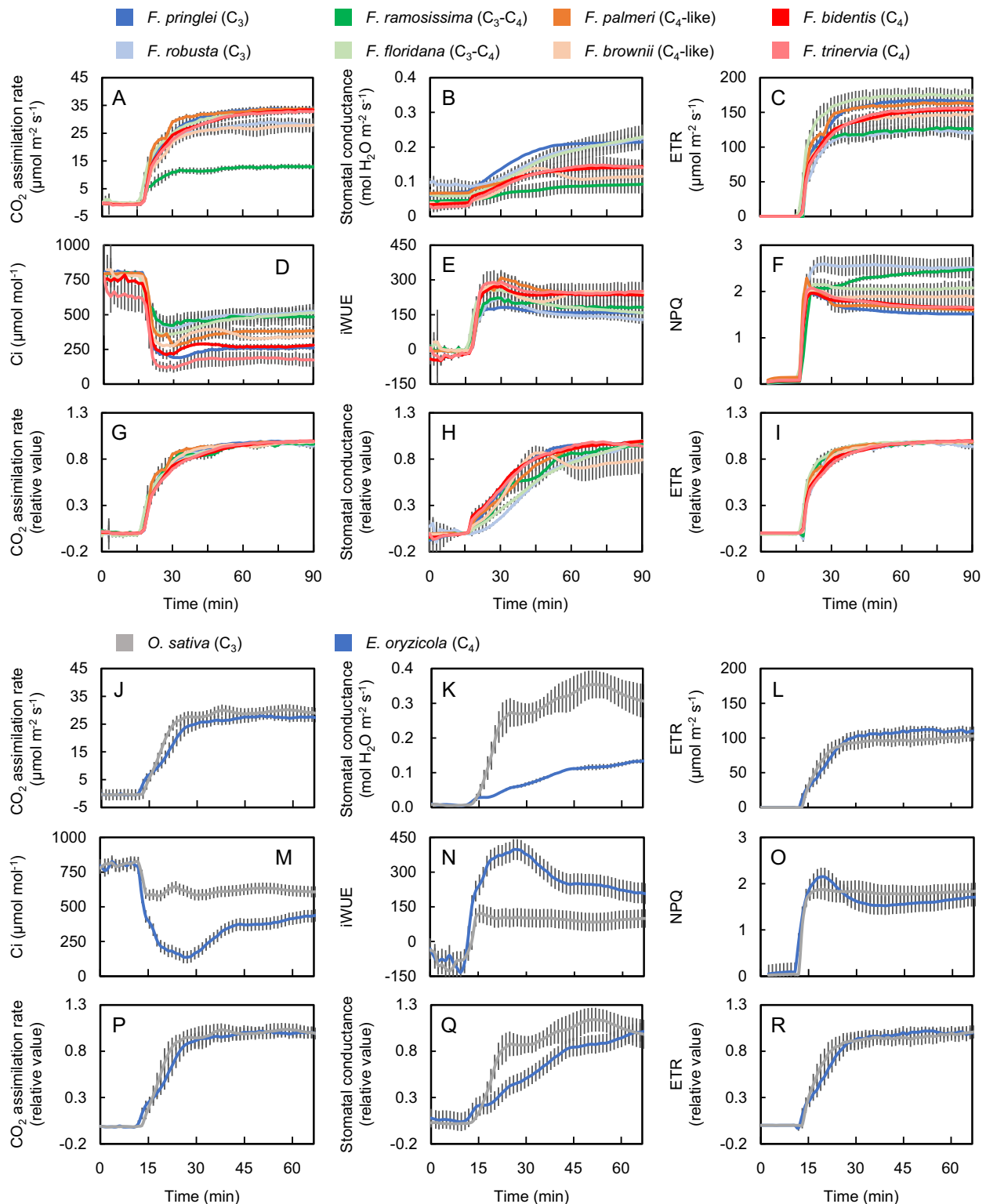


FIGURE 3 Induction responses of gas exchange and chlorophyll fluorescence parameters at $800 \mu\text{mol CO}_2 \text{ mol}^{-1}$ in (A–I) C_3 , C_3 – C_4 , C_4 -like and C_4 dicot species and in (J–R) C_3 and C_4 monocot species after step increases in light from darkness to $\text{PPFD} = 1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ over 75 min. (A, J) CO_2 assimilation rate (A), (B, K) stomatal conductance (g_s), (C, L) electron transport rate (ETR), (D, M) intercellular CO_2 concentration (C_i), (E, N) intrinsic water use efficiency (iWUE), (F, O) non photochemical quenching (NPQ). Equation 1 was used to estimate relative values of (G, P) A, (H, Q) g_s , and (I, R) ETR. Data are means \pm SE ($n = 4$ –5).

We also calculated t_{50} and t_{90} for the induction responses among the dicot group per genotype at $400 \mu\text{mol CO}_2 \text{ mol}^{-1}$ (Figure S2) and at $800 \mu\text{mol CO}_2 \text{ mol}^{-1}$ (Figure S3). There were

significant differences in t_{50} for g_s and ETR (Figure S2B, C), and there were also differences in t_{90} for A and g_s at $400 \mu\text{mol CO}_2 \text{ mol}^{-1}$ (Figure S2A, B). At $800 \mu\text{mol CO}_2 \text{ mol}^{-1}$, there were no

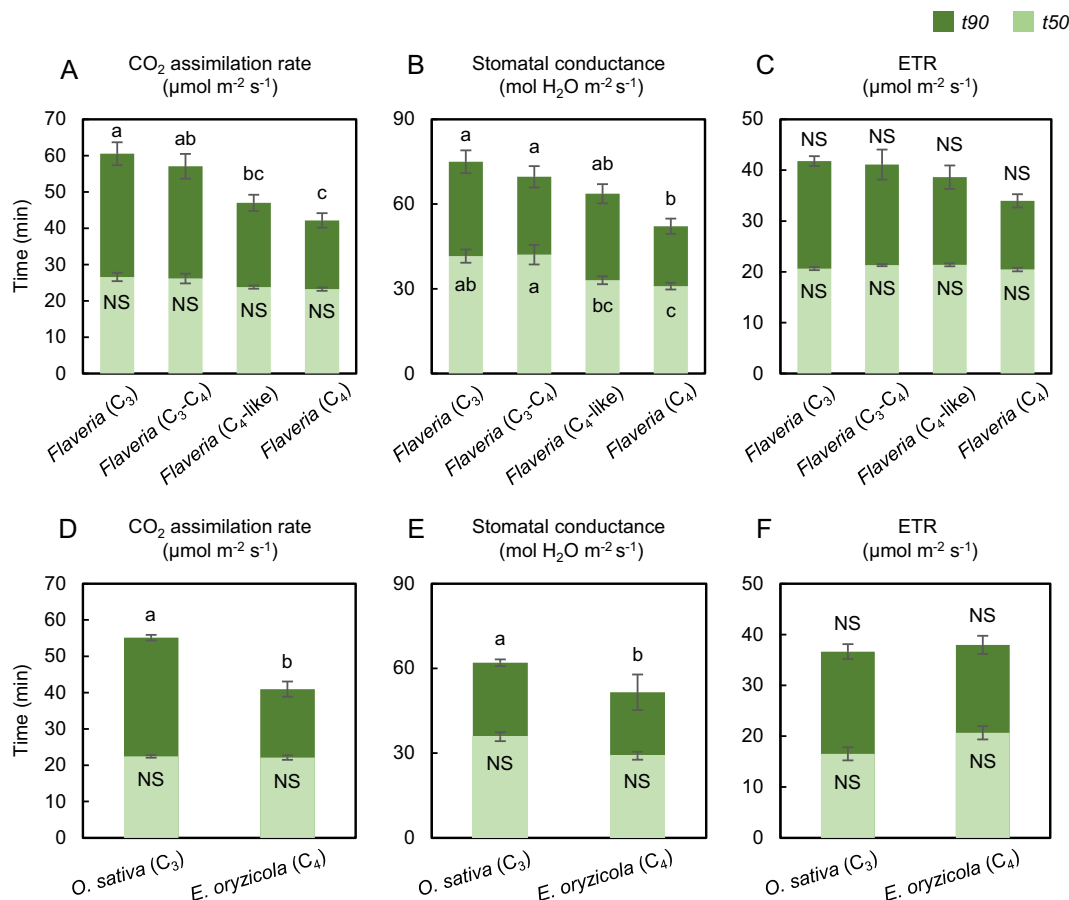


FIGURE 4 Induction kinetics of CO₂ assimilation rate (A), stomatal conductance (g_s) and electron transport rate (ETR) at 400 $\mu\text{mol CO}_2 \text{mol}^{-1}$ in (A-C) C₃, C₃-C₄, C₄-like and C₄ dicot species and (D-F) C₃ and C₄ monocot species. Times when values reached 50% (t_{50} ; pale green) and 90% (t_{90} ; deep green) of maximum values were compared among C₃ (*Flaveria pringlei*, *F. robusta*), C₃-C₄ (*F. ramosissima*, *F. floridana*), C₄-like (*F. palmeri*, *F. brownii*) and C₄ dicots (*F. bidentis*, *F. trinervia*) and between C₃ (*Oryza sativa*) and C₄ (*Echinochloa oryzicola*) monocots. t_{50} and t_{90} were estimated with the data in Figure 2. Columns with the same letter are not significantly different at $P < 0.05$ by Tukey-Kramer test. Data are means \pm SE, $n = 8-10$.

significant variations in t_{50} or t_{90} for A and t_{50} for g_s (Figure S3A, B), but there are variations in t_{90} for g_s and t_{50} and t_{90} for ETR (Figure S3B, C).

3.3 | Relationship between photosynthetic induction, light-induced stomatal dynamics, and CO₂ compensation points

From the A-C_i curves, we estimated the CO₂ compensation point as a parameter of the CO₂-concentrating mechanism in the *Flaveria* species. Values were significantly lower in C₄ and C₄-like plants than in C₃ and C₃-C₄ plants, and in C₃-C₄ plants than in C₃ plants (Figure 6). At 400 $\mu\text{mol CO}_2 \text{mol}^{-1}$ the CO₂ compensation points were significantly correlated with t_{90} of A and g_s (Figure 6A, B), and t_{90} of A was significantly correlated with t_{90} of g_s (Figure 6C). On the other hand, at 800 $\mu\text{mol CO}_2 \text{mol}^{-1}$, there were no significant correlations.

3.4 | Intrinsic WUE at ambient and elevated CO₂ conditions

There were large variations in steady-state iWUE among plants at both 400 and 800 $\mu\text{mol CO}_2 \text{mol}^{-1}$ (Figure S4). iWUE in all plants was higher at 800 $\mu\text{mol CO}_2 \text{mol}^{-1}$ than at 400 $\mu\text{mol CO}_2 \text{mol}^{-1}$ (Figure 7) and was significantly greater in C₄ and C₄-like plants compared to C₃ and C₃-C₄ plants at both CO₂ concentrations (Figure 7).

4 | DISCUSSION

4.1 | C₄ photosynthesis can use fluctuating light more efficiently than C₃ photosynthesis

Through a comparison of 10 species, we found that photosynthetic induction and light-induced stomatal dynamics of C₄ species and their

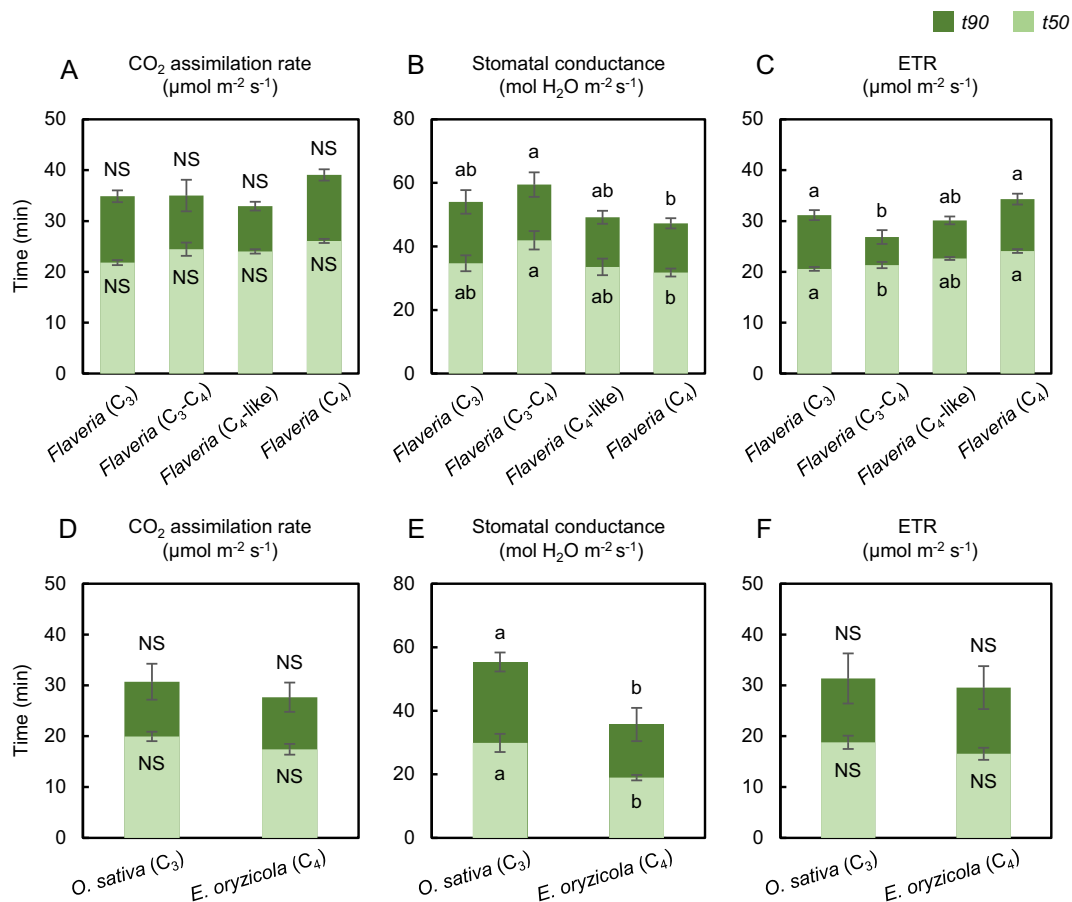


FIGURE 5 Induction kinetics of CO₂ assimilation rate (A), stomatal conductance (g_s) and electron transport rate (ETR) at 800 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ in (A-C) C₃, C₃-C₄, C₄-like and C₄ dicot species and (D-F) C₃ and C₄ monocot species. Times when values reached 50% (t_{50} ; pale green) and 90% (t_{90} ; deep green) of maximum values were compared among C₃ (*Flaveria pringlei*, *F. robusta*), C₃-C₄ (*F. ramosissima*, *F. floridana*), C₄-like (*F. palmeri*, *F. brownii*) and C₄ dicots (*F. bidentis*, *F. trinervia*) and between C₃ (*Oryza sativa*) and C₄ (*Echinochloa oryzicola*) monocots. t_{50} and t_{90} were estimated with the data in Figure 3. Columns with the same letter are not significantly different at $P < 0.05$ by Tukey-Kramer test. Data are means \pm SE, $n = 8-10$.

close relatives are more rapid than those of C₃ species at a CO₂ concentration of 400 $\mu\text{mol mol}^{-1}$ (Figures 2, 4), but not at 800 $\mu\text{mol mol}^{-1}$ (Figures 3, 5). These findings agree with earlier studies reporting that the rapidity of g_s is greater in monocots than dicots and that C₄ species are faster than C₃ species (McAusland et al., 2016; Israel et al., 2022). These findings clearly show that at the current CO₂ concentration, both monocot and dicot C₄ species and their close relatives can harness fluctuating light more efficiently than C₃ species. This efficiency is partly due to their CO₂ concentrating mechanisms and adaptation to fluctuating light environments (Lee et al., 2021).

Some studies have suggested that C₄ species have lower light-use efficiency than C₃ species under fluctuating light (e.g., Kubásek et al., 2013; Li et al., 2021), while others have reported that there is no inherent limitation on the ability of C₄ species to use sunflecks (brief periods of direct high intensity sunlight) relative to C₃ species (Percy et al., 1985; Chazdon and Percy, 1986; Watling et al., 1997; Sage, 2014). These disparities may stem from differences in the pattern of the fluctuating light and the assessment of such responses, including the specific parameters used to quantify differences

between plants/species. Studies using longer-duration fluctuating light, with intervals of 1 to 15 min, have suggested that C₄ plants can perform just as well or even outperform C₃ species (Percy and Calkin, 1983; Stitt & Zhu, 2014; Slattery et al., 2018) which agree with our findings reported here. In contrast, when using short-duration sunflecks lasting only a few seconds, C₄ plants may be less efficient than C₃ plants (Krall and Percy, 1993; Kubásek et al., 2013). The pattern of light fluctuations and intensity of “flecks” varies greatly between forest understorey and open field conditions (Kimura et al., 2020), and they also varies between open field of cropland and understorey of it (Durand et al., 2021). There are quite differences even in fellow open fields in accordance with their environments (e.g. place, season or planted species). In the understorey, light remains below the light compensation point and exerts minimal influence on photosynthesis in almost two-third of the day time (Liu et al., 2015). In these conditions sunflecks can contribute between 30%–80% of the carbon gain in understorey plants (Percy, 1987, 1988, 1990). In contrast, under open field conditions, plants are consistently exposed to fluctuations between low and high light intensities, although these

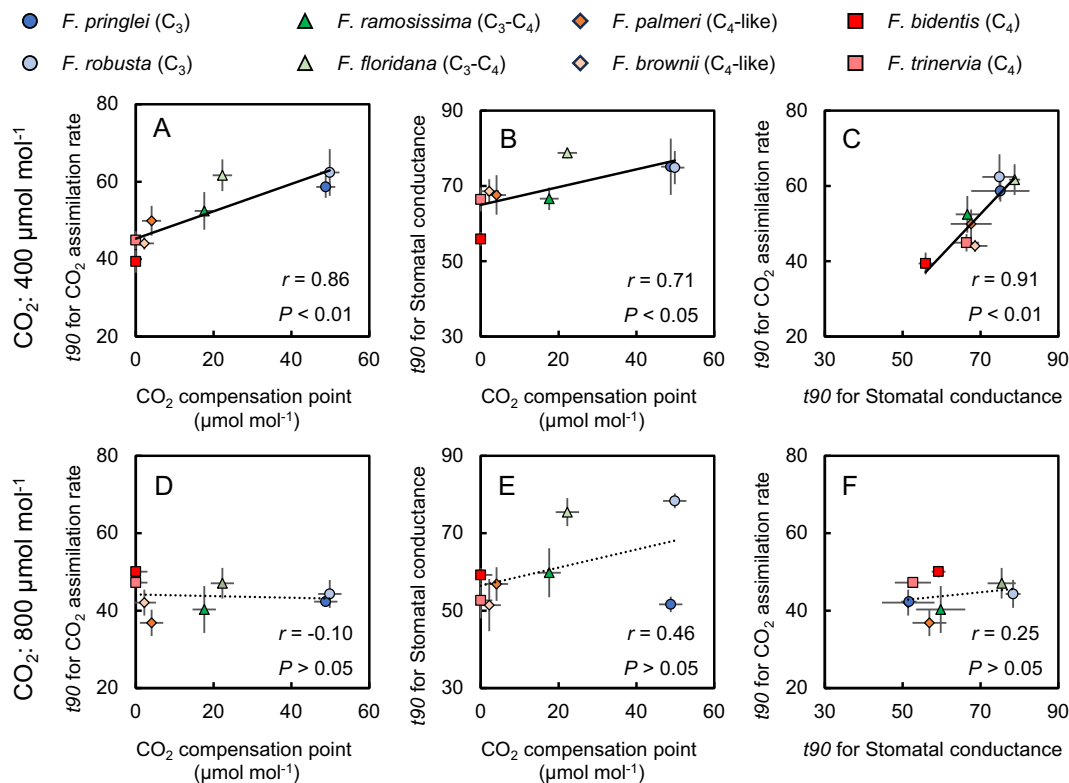


FIGURE 6 Relationships among induction kinetics and CO₂ compensation points in C₃, C₃-C₄, C₄-like and C₄ dicot and C₃ and C₄ monocot species. Panels show correlations between CO₂ compensation points and the time when CO₂ assimilation rate (A) or stomatal conductance (g_s) reached 90% (t₉₀) of maximum values, and between t₉₀ of CO₂ assimilation rate and t₉₀ of g_s at 400 μmol CO₂ mol⁻¹ and (D-F) 800 μmol CO₂ mol⁻¹ in C₃ (*Flaveria pringlei*, *F. robusta*), C₃-C₄ (*F. ramosissima*, *F. floridana*), C₄-like (*F. palmeri*, *F. brownii*) and C₄ dicots (*F. bidentis*, *F. trinervia*) and C₃ (*Oryza sativa*) and C₄ (*Echinochloa oryzicola*) monocots. Data are means ± SE, n = 4–5. Solid lines indicate significant correlations.

intensities, in general, tend to be higher than those of the understory (Percy, 1990).

C₃ and C₄ plants are exposed to various patterns of fluctuating light depending on their habitat. In open field conditions where high-intensity light periods are common, C₄ plants could more effectively harness the energy from fluctuating light compared to C₃ plants. However, C₄ plants may face challenges in environments where consistently low light is intermittently disrupted by brief periods of high-intensity sunflecks, as commonly found in the understory.

4.2 | Light-induced stomatal dynamics contributed to rapid photosynthetic induction in C₄ plants

In both C₃ and C₄ plants, photosynthesis is induced over several minutes as a dark- or low light- adapted leaves are exposed to higher light intensity (Figures 2, 3). This photosynthetic induction response is determined mainly by biochemical and stomatal limitations (Percy 1990; Way and Percy, 2012; Lawson et al., 2010; 2012; Yamori, 2016). Our results show that photosynthetic induction at 400 μmol CO₂ mol⁻¹ was more rapid in C₄ and C₄-like than in C₃ monocots and dicots (Figures 2, 4). The rapid increase in A was

accompanied with a rapid g_s induction, indicating that the rapid stomatal responses in the C₄ and C₄-like species removed diffusional constraints on photosynthesis, facilitating rapid induction of A (Lawson & Blatt, 2014). The positive relationship between t₉₀ of A and t₉₀ of g_s at 400 μmol CO₂ mol⁻¹ (Figure 6C), but not at 800 μmol CO₂ mol⁻¹ (Figure 6F), at which the CO₂ supply rarely limits photosynthesis (Shimadzu et al., 2019), indicating that faster light-induced stomatal dynamics was responsible for the faster photosynthetic induction response in C₄ species rather than biochemical limitation. Our findings are supported by a recent report of more rapid stomatal opening and closure in five C₄ crops compared with four C₃ crops (Ozeki et al., 2022). It is well established that C₄ plants have greater stomatal sensitivity to C_i than C₃ plants (Dubbe et al., 1978; Sharkey and Raschke, 1981; Ramos and Hall, 1982; Huxman and Monson, 2003). These inherent properties likely contribute to the rapid dynamics of stomatal responses and, consequently, of photosynthetic induction in C₄ plants (Figures 2, 4). The difference of iWUE supports the effect of stomatal dynamics to photosynthetic induction. At both 400 μmol CO₂ mol⁻¹ and 800 μmol CO₂ mol⁻¹, C₄ and C₄-like plants showed higher iWUE than C₃ and C₃-C₄ plants (Figure 7). This indicates that C₄ and C₄-like plants reduce unnecessary water loss by rapid stomatal response. This rapid stomatal response at 400 μmol CO₂ mol⁻¹ is related to K⁺ channel response (Silva-Alvim

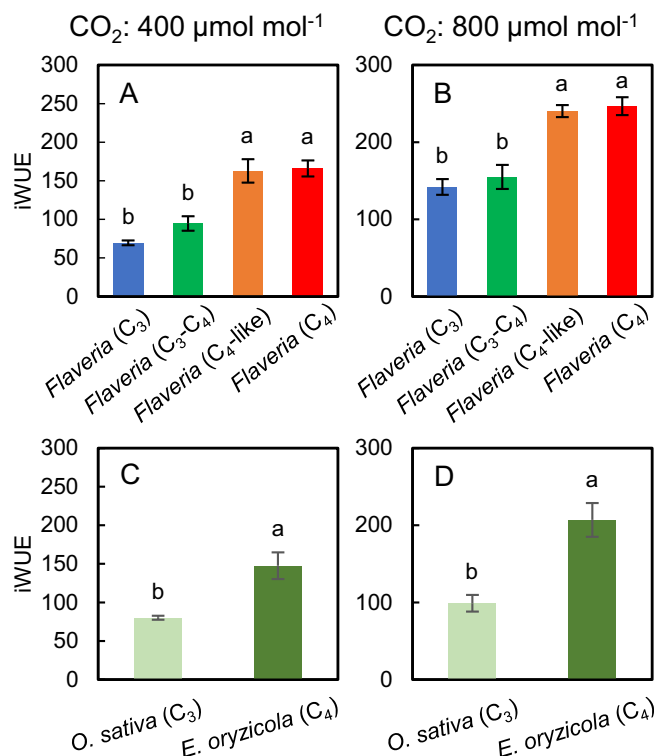


FIGURE 7 Comparison of intrinsic water use efficiency (iWUE) among (A, B) C₃, C₃-C₄, C₄-like and C₄ dicots, and (C, D) C₃ and C₄ monocots at 400 and 800 μmol CO₂ mol⁻¹. Dicots: C₃ (*Flaveria pringlei*, *F. robusta*), C₃-C₄ (*F. ramosissima*, *F. floridana*), C₄-like (*F. palmeri*, *F. brownii*) and C₄ (*F. bidentis*, *F. trinervia*). Monocots: C₃ (*Oryza sativa*) and C₄ (*Echinochloa oryzicola*). Values were obtained from the data in Figures 2 and 3. Columns with the same letter are not significantly different at $P < 0.05$ by Tukey-Kramer test. Data are means \pm SE, $n = 8-10$.

et al., 2024), but the relationship between stomatal response and K⁺ channel response at 800 μmol CO₂ mol⁻¹ is unclear. So further research to measure K⁺ channel response at various CO₂ concentrations is needed.

The CO₂-concentrating mechanism provides C₄ plants advantages in CO₂-limited conditions such as high temperature, drought conditions. C₄ plants keep a high CO₂ assimilation rate in such a condition due to high intercellular CO₂ concentration with limited stomatal aperture, and this limited stomatal closure maintains water use efficiency. In addition, differences in the photosynthetic responses to fluctuating light between C₃ and C₄ plants can be attributed to the C₄ plants' CO₂-concentrating mechanism (Figure 6A, C). The CO₂ compensation point serves as a reliable indicator of the degree of C₄ photosynthesis, with a lower CO₂ compensation point signifying a higher degree of C₄ photosynthesis (e.g., Ku et al., 1991). The positive relationship between t_{90} for A and the CO₂ compensation point at 400 μmol CO₂ mol⁻¹ (Figure 6A), but not at 800 μmol CO₂ mol⁻¹ (Figure 6D), implies that the CO₂-concentrating mechanism may alleviate the limitation of CO₂ supply during the photosynthetic induction response in C₄ plants. This proposition is substantiated

by previous reports that C₄ species have an advantage of photosynthetic induction due to reduced stomatal constraints in the build-up of intercellular CO₂ (Usuda and Edwards, 1984; Furbank and Walker, 1985).

In summary, our results show that both C₄ monocots and C₄ dicots show rapid photosynthetic induction due to quick g_s responses and CO₂-concentrating mechanisms, unlike C₃ plants. Consequently, C₄ plants are less susceptible to the challenges of fluctuating light in natural field conditions, at 400 μmol CO₂ mol⁻¹. However, this relationship changes at 800 μmol CO₂ mol⁻¹. Further research is required to determine the extent of limitation imposed by photosynthetic enzymes during photosynthetic induction in C₄ plants, the effect of guard cell shape and effect of K⁺ channel response at 800 μmol CO₂ mol⁻¹.

AUTHOR CONTRIBUTIONS

W.Y., R.S. and T.L. conceived and designed the experiments. Q.Y. and N.K. grew plants. K.T., Q.Y., N.K., K.S. and Y.W. performed the experiments and analysed the data. K.T., Q.Y. and N.K. prepared figures, and K.T., Q.Y., N.K., W.Y., R.S. and T.L. prepared the manuscript. All authors have read and approved the final version of this manuscript.

ACKNOWLEDGEMENTS

We thank Mr Fu Hao for his support to measure photosynthesis.

FUNDING INFORMATION

This work was supported by KAKENHI grants to W.Y. (18KK0170, 20H05687, 21H02171, 24A301 and 24H02277) from the Japan Society for the Promotion of Science (JSPS).

DATA AVAILABILITY STATEMENT

Supporting data can be requested by contacting the corresponding author.

ORCID

Keiichiro Tanigawa <https://orcid.org/0009-0002-2888-1199>

Naoya Katsuhama <https://orcid.org/0000-0003-2395-837X>

Kazuma Sakoda <https://orcid.org/0000-0002-7373-7105>

Tracy Lawson <https://orcid.org/0000-0002-4073-7221>

Wataru Yamori <https://orcid.org/0000-0001-7215-4736>

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SUPPORTING INFORMATION

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

How to cite this article: Tanigawa, K., Yuchen, Q., Katsuhama, N., Sakoda, K., Wakabayashi, Y., Tanaka, Y. et al. (2024) C₄ monocots and C₄ dicots exhibit rapid photosynthetic induction response in contrast to C₃ plants. *Physiologia Plantarum*, 176(4), e14431. Available from: <https://doi.org/10.1111/ppl.14431>