During photosynthetic induction, biochemical and stomatal limitations differ between Brassica crops

Taylor, Samuel H.¹*, Orr, Douglas J. ¹, Carmo-Silva, Elizabete¹, and Long, Stephen P. ¹,²

¹Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK
²Departments of Plant Biology and of Crop Sciences, Carl R. Woese Institute of Genomic Biology, University of Illinois, 1206 W. Gregory Dr., Urbana, IL 61801, USA

Running title: Photosynthetic induction in Brassica crops

*corresponding author: s.taylor19@lancaster.ac.uk
Abstract

Interventions to increase crop radiation use efficiency rely on understanding how biochemical and stomatal limitations affect photosynthesis. When leaves transition from shade to high light, slow increases in maximum Rubisco carboxylation rate and stomatal conductance limit net CO$_2$ assimilation for several minutes. However, as stomata open, intercellular [CO$_2$] increases, so electron transport rate could also become limiting. Photosynthetic limitations were evaluated in three important Brassica crops: B. rapa, B. oleracea and B. napus. Measurements of induction after a period of shade showed that net CO$_2$ assimilation by B. rapa and B. napus saturated by 10 min. A new method of analyzing limitations to induction by varying intercellular [CO$_2$] showed this was due to co-limitation by Rubisco and electron transport. By contrast, in B. oleracea, persistent Rubisco limitation meant that CO$_2$ assimilation was still recovering 15 min after induction. Correspondingly, B. oleracea had the lowest Rubisco total activity. The methodology developed, and its application here, shows a means to identify the basis of variation in photosynthetic efficiency in fluctuating light, which could be exploited in breeding and bioengineering to improve crop productivity.

Key words

Brassica oleracea, Brassica napus, Brassica rapa, dynamic photosynthesis, Rubisco, photosynthetic electron transport, photosynthetic induction, stomata, crop improvement, CO$_2$ response
Introduction

The continued growth of the global human population and its increasing urbanisation will lead to increased pressure on farming systems over the next half century, and increased productivity on the land we are already using will be crucial to minimize the environmental impacts (Tilman, Balzer, Hill & Befort 2011). In this context, it is essential to understand photosynthetic efficiency because it fundamentally affects the productivity and efficiency of resource use by crops. The majority of crops use C₃ photosynthesis, which requires massive investment of nitrogen in leaf chloroplasts, where 21-74% of leaf soluble protein is allocated to the primary CO₂ fixing enzyme ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco; Carmo-Silva, Scales, Madgwick & Parry 2015). Furthermore, a cost of allowing CO₂ into the leaf for photosynthesis, is the escape of water vapour via transpiration (Farquhar & Sharkey 1982; Raschke 1975). Consequently, crop biological N₂ fixation and crop applied N fertilisers now account for more than 44% of the total annual N entering the global biosphere (Fowler et al. 2013), and crop irrigation accounts for 70% of annual global human water use (Haddeland et al. 2014).

The major focus of studies of crop photosynthetic efficiency has been under light-saturating steady-state conditions. Yet these are rare for crop leaves in the field or glasshouse. Importantly, crop photosynthetic efficiency may be substantially affected by dynamic regulation in non-steady-state conditions. Adjustments to cope with changes in availability of light, e.g., caused by temporary shading within crop canopies, result in deviation from performance optima that are measured and defined in terms of steady-state conditions (Kaiser et al. 2016; Kromdijk et al. 2016; Lawson & Vialet-Chabrand, 2019; Morales et al. 2018; Tanaka, Adachi & Yamori, 2019; Taylor & Long 2017; Wang, Burgess, de Becker & Long 2020; Zhu, Ort, Whitmarsh & Long 2004). The effects of non-steady-state conditions on
photosynthetic efficiency, including the effects of temporary shade, remain poorly characterised for a great many crop species.

Currently, a leading strategy for increasing crop efficiency is to improve radiation use efficiency (Ort et al. 2015; Zhu, Long & Ort 2010). A key area of progress is improving the speed at which photosynthesis responds to dynamic variation in sun and shade. Slow relaxation of non-photochemical quenching (NPQ) during sun-shade transitions is one factor that limits crop radiation use efficiency (Zhu et al. 2004), and speeding up this process has been shown to increase plant productivity (Kromdijk et al. 2016). Slow induction of photosynthesis during shade-sun transitions is also potentially important (Kaiser et al. 2015; Pearcy, Krall & Sassenrath-Cole 1996). Evidence suggests that slow induction significantly decreases diurnal CO₂ assimilation, and/or that there is significant genetic variation in rates of induction amenable to breeding in wheat (Salter, Merchant, Richards, Trethowan & Buckley 2019; Taylor & Long 2017), rice (Acevedo-Siaca et al. 2020; Yamori, Masumoto, Fukayama & Makino 2012), cassava (De Souza, Wang, Orr, Carmo-Silva & Long 2020), and soya (Soleh et al. 2016; Wang et al. 2020). However, dynamic changes in the components of non-stomatal limitations affecting photosynthesis during shade-sun transitions have been characterised infrequently, so it remains unclear whether interventions that target specific biochemical processes limiting induction of photosynthesis, e.g., increasing rates of Rubisco activation (Yamori et al. 2012), will be similarly effective in a broad range of crop species.

For C3 leaves, supply of CO₂ mediated by stomatal conductance (Farquhar & Sharkey, 1982) results in net CO₂ assimilation rate (A)-intercellular [CO₂] (cᵢ) relationships (A/cᵢ responses) that are expected to be controlled by different biochemical limitations depending on cᵢ. At high light and lower cᵢ, photosynthesis is usually limited by maximum rates of RuBP carboxylation by Rubisco (V₉₈₅), but above a threshold cᵢ (cᵢ, trans) RuBP regeneration resulting from Calvin Benson Cycle turnover, driven principally by rates of
electron transport ($J$) becomes limiting (von Caemmerer & Farquhar 1981; Farquhar, von Caemmerer & Berry, 1980). Robert Pearcy and colleagues first extended this model to photosynthetic induction during the 1980s (reviewed in Pearcy et al. 1996), and their dynamic $A/c_i$ method (Chazdon & Pearcy, 1986) remains a gold standard for analysing biochemical limitation during shade-sun transitions (Acevedo-Siaca et al. 2020; De Souza et al. 2020; Salter et al. 2019; Soleh et al. 2016; Taylor & Long, 2017). The dynamic $A/c_i$ approach consists of a series of inductions measured at different [CO$_2$]s. Early applications provided evidence that, subsequent to a 1-2 min RuBP-regeneration limited ‘fast-phase’ (Sassenrath-Cole & Pearcy 1992), slow increases in both $V_{c,\text{max}}$ and $g_s$ are key controls affecting the rate at which $A$ recovers following shade (Chazdon & Pearcy, 1986; Kirschbaum & Pearcy, 1988). This understanding facilitated subsequent work addressing the function of Rubisco activase ($Rca$), which drives increases in $V_{c,\text{max}}$ during induction (Carmo-Silva & Salvucci, 2013; Hammond, Andrews, Mott & Woodrow 1998; Woodrow & Mott, 1989), and the assumption of persistent $V_{c,\text{max}}$ limitation during induction has recently been used to improve methods for analysing biochemical and stomatal limitations during induction (Deans, Farquhar & Busch 2019a).

Despite their importance, a caveat of published dynamic $A/c_i$ measurements is potential feedback between $c_i$ and photosynthetic induction: greater $c_i$ following shade is linked with faster induction (Kaiser, Kromdijk, Harbinson, Heuvelink & Marcelis, 2017; Kirschbaum & Pearcy 1988; Woodrow, Kelly & Mott 1996). Because this effect could inflate apparent rates of increase in $V_{c,\text{max}}$ obtained from dynamic $A/c_i$ experiments, and underestimate absolute effects of $V_{c,\text{max}}$ on induction, alternative protocols that establish the dynamic behaviour of $V_{c,\text{max}}$ without holding leaves at different [CO$_2$]s for extended periods can better establish impacts on crop performance.
The $[\text{CO}_2]$ denoting the transition from limitation by $V_{c,\text{max}}$ to limitation by $J$ on the $A/c_i$ response ($c_{i,\text{trans}}$) is an important parameter for understanding photosynthetic efficiency. Atmospheric $[\text{CO}_2]$ is higher today than at any stage since domestication of crop plants began (Indermühle et al. 1999; Larson et al. 2014; Sage 1995). Therefore, limitation by $V_{c,\text{max}}$ because of low $c_i$ is likely to have been an important constraint on crop photosynthesis, including photosynthetic induction, throughout the history of agriculture. Today and in the future, however, higher ambient $[\text{CO}_2]$ and/or increasing nitrogen limitation (which diminishes $V_{c,\text{max}}$ and $J$) may result in more frequent limitation of $A$ by $J$, including under saturating light conditions where $V_{c,\text{max}}$ would previously have been the primary biochemical control (Long, Ainsworth, Rogers & Ort 2004; Kromdijk & Long 2016). Whether the operating point for $A$ falls at, or towards higher or lower $c_i$ than $c_{i,\text{trans}}$, will impact photosynthetic optimisation and therefore efficiency of resource use under steady state conditions. Photosynthesis at or close to $c_{i,\text{trans}}$ implies balanced Calvin Benson Cycle function, maximizing returns on investment towards RuBP carboxylation and regeneration capacity (von Cammerer & Farquhar, 1981; Farquhar & Sharkey, 1982; Long et al. 2004; Kromdijk & Long 2016). Because the dynamic $A/c_i$ method enables $c_{i,\text{trans}}$ to be determined under non-steady-state conditions (Taylor & Long 2017) and establishes the patterns and impacts of changes in $V_{c,\text{max}}$ and $J$, dynamic $A/c_i$ measurements can provide unique mechanistic insights into deviations from optimal photosynthesis during induction.

Crops from the genus *Brassica* (L.) are key sources of vitamins and minerals globally (Rakow 2004) and provide interesting physiological contrasts. *Brassica* can differ considerably in terms of e.g., leaf size and thickness, and may be annual or biennial, which would be expected to drive alternative leaf structural and biochemical investments (Wright et al. 2004). The origins and inter-relationships between *Brassica* species are well understood (Liu et al. 2014; Parkin et al. 2005; Rana et al. 2004). From the perspective of understanding
how induction varies among crop accessions, the relationship between *B. oleracea* (L.), *B. rapa* (L.), and their allotetraploid hybrid *B. napus* (L.) is particularly interesting. Divergence between *B. oleracea* and *B. rapa* occurred as much as 4 Mya (Inaba & Nishio 2002), and *B. napus* most likely originated in agricultural settings, i.e., < 10 kya (Rana *et al.* 2004). Consequently, gene families from both *B. oleracea* and *B. rapa* that are present in the allotetraploid *B. napus* genome (Rana *et al.*, 2004), may include those specifying the small subunit of Rubisco and *Rca*. Their evolutionary history, therefore, makes these three species an interesting test of the extent to which fairly close relatives can show differentiation in non-steady-state photosynthesis, especially the impacts of *V_{c,max}* on induction.

Using gas exchange and chlorophyll fluorescence, limitations affecting steady-state and non-steady-state photosynthesis were determined for *B. oleracea*, *B. napus* and *B. rapa*. 1) Steady-state leaf gas exchange was used in combination with biochemistry of leaf extracts to determine whether photosynthetic characteristics, including the predominant biochemical limitation, differed. 2) Gas exchange time-series for induction measured at ambient [CO₂] were used to establish whether there were differences in terms of: fast- (before 2 min) and slow- (after 2 min) phases of induction, as well as periods dominated by non-stomatal factors, which include biochemistry (decreasing *c_i*), or effects of increasing *g_s* (increasing *c_i*). 3) Apparent biochemical limitations during induction were established in detail using a new dynamic *A/c_i* response methodology, designed to overcome a key caveat of previous experiments by not holding leaves at sub- or super-ambient [CO₂]s for extended periods.

**Materials and Methods**

**Plant material**

The three *Brassica* were represented by: a commercial winter oil seed rape, *B. napus* cv. Elgar (Elsoms Seeds Ltd. Spalding, UK); Yellow Sarson, *B. rapa* ssp. *trilocularis* genotype
R-o-18, which has a similar developmental ontogeny to oilseed rape (Stephenson et al. 2010); and Gai lan, *B. oleracea* ssp. *alboglabra*, genotype A12DHd (R-o-18 and A12DHd, Warwick Crop Centre, Wellesbourne, UK).

Plants used for gas exchange measurements grew in controlled environment greenhouses set to maintain day/night temperatures at 24/18 °C. A 16 h daylength was maintained using supplementary lighting from high pressure sodium lamps (SON-T 400W, Philips Lighting, Eindhoven NL) that provided a photosynthetic photon flux density (PPFD) of ~500 μmol m$^{-2}$ s$^{-1}$ at canopy level if external short-wave irradiance decreased below 250 W m$^{-2}$ (~570 μmol m$^{-2}$ s$^{-1}$ PPFD). Seedlings were germinated in 40 mL cells (PG Mix, Yara, Grimsby, UK), and were transplanted to 1.5 L pots one week after emergence, in each case using a soil-less compost mix (Petersfield Products, Leicester, UK) that incorporated a broad range fertilizer. Checks were made daily to ensure that compost was kept moist without overwatering.

Plants used for biochemistry were also sown, germinated and transplanted to 1.5 L pots in the greenhouse, containing the same compost mix as above. They were then transferred into controlled environment cabinets (Microclima 1750, Snijders Scientific B.V., Netherlands) two weeks after transplanting. Cabinets were set to maintain day/night temperatures at 25/15 °C, RH was maintained at ~60%, and a 16 h daylength was achieved with canopy-level PPFD ~450 μmol m$^{-2}$ s$^{-1}$. Each species was sampled in five repeats of the experiment: four plants per species were transferred to the controlled environment cabinet, and after ~24 d in the cabinet, one leaf disc (0.55 cm$^2$) per plant was taken from the youngest fully expanded leaf and immediately snap frozen in liquid N$_2$. To average out the effects of plant-to-plant variation, within each of the five batches of plants, the four discs per species were pooled for the Rubisco content and activity analyses described below.
Steady state photosynthesis

Measurements were made 5-6 weeks after planting for *B. rapa* and *B. napus*, and one or two weeks later for the slower growing *B. oleracea*. Recently expanded leaves were enclosed in the controlled environment cuvette of a photosynthesis system (LI-6800F, LI-COR, Lincoln NE, USA), which incorporates open-path infra-red CO$_2$ and H$_2$O analysers, and an integrated modulated fluorometer/light source. Leaf temperature was controlled at 25 °C, and leaf-air vapour pressure deficit (VPD$_{leaf}$) at 1.2 kPa. To measure photosynthetic responses to PPFD, leaves were brought to steady-state (stable $A$ and stomatal conductance to water ($g_{sw}$) over 5 min) at a PPFD of 1500 μmol m$^{-2}$ s$^{-1}$ and [CO$_2$] of 392 ± 3.5 μmol mol$^{-1}$ (mean ± sd; reference channel 430 μmol mol$^{-1}$). PPFD was then varied to supply 2000, 1800, 1500, 1200, 1000, 800, 600, 500, 400, 300, 250, 200, 150, 100, 50, and 0 μmol m$^{-2}$ s$^{-1}$ inside the cuvette. Measurements were taken as soon as $A$ stabilised at each PPFD. Leaves were brought back to steady-state under the initial conditions, then the steady-state response of $A$ to $c_i$ was determined using measurements at different reference CO$_2$ concentrations: firstly, 430, 300, 200, 150, 100, 50, and ~ 0 μmol mol$^{-1}$, then, after return to steady state at 430 μmol mol$^{-1}$; 500, 600, 700, 900, 1000, and 1200 μmol mol$^{-1}$. In addition to gas exchange parameters calculated following von Caemmerer and Farquhar (1981), measurements during CO$_2$ response curves captured steady state ($F_s$) and maximum ($F_{m'}$) fluorescence yields using a multiphase flash, allowing use of the effective quantum yield [$\Phi_{PSII} = (F_{m'} - F_s)/F_{m'}$] as an additional indicator of photosynthetic limitation-state based on its proportionality with $J$ (e.g., Gu *et al.* 2010, Busch & Sage 2017; Supplementary Fig. 1).

Photosynthetic induction

Photosynthetic induction responses at ambient [CO$_2$] were measured by establishing steady-state gas exchange at: PPFD, 1500 μmol m$^{-2}$ s$^{-1}$; reference [CO$_2$], 430 μmol mol$^{-1}$; cuvette...
air temperature, 25 °C; and cuvette RH 65% (VPD_{leaf} 1.08 ± 0.075 kPa). A shade fleck was then simulated by a step decrease in PPFD to 150 μmol m^{-2} s^{-1} for 30 min, followed by a step increase back to 1500 μmol m^{-2} s^{-1}. Gas analysers were matched one minute before starting the sun-shade-sun sequence, and measurements were logged every 10 s from one min before shade until at least 28 min after shade.

The following key timesteps from the 10 s resolution induction curves were identified. First, the end of the RuBP regeneration dominated ‘fast-phase’ of induction was taken to be 2 min after the return to high light, following shade. Second, t_{ci,min} was the time at which minimum c_i was observed during induction, marking the transition between predominant limitation by non-stomatal factors (which results in decreasing c_i) and increasing stomatal conductance (g_s; which results in increasing c_i). Next, t_{A,90} was the timepoint at which A had recovered 90% of the difference [A pre-shade − A end shade]. Using these timepoints, recovery in A, as a proportion of [A pre-shade − A shade], was attributed to the fast-phase (R_{fast}), non-stomatal dominated (R_{ci,min}), and non-stomatal dominated recovery not attributable to the fast-phase (R_{ci,min} − R_{fast}), i.e., slow phase non-stomatal recovery. The duration of recovery dominated by effects of g_s was approximated by t_{A,90} − t_{ci,min}.

**Dynamic A/c_i measurements**

To characterise changes in factors limiting photosynthesis during shade-sun transitions, a dynamic A/c_i method was implemented that improved on previously published versions (Acevedo-Siaca et al. 2020; Chazdon & Pearcy 1986; De Souza et al. 2020; Salter et al. 2019; Soleh et al. 2016; Taylor & Long 2017) by removing the potentially confounding effect of extended incubation in various [CO_2]s. Leaves were first brought to steady state under the same conditions as for measurements of photosynthetic induction described above. A 30 min period of shade was then imposed using a PPFD of 100 μmol m^{-2} s^{-1}. Following
Taylor & Long (2017), to prevent stomatal closure in response to this shade by maintaining $c_i$ at approximately twice the compensation point (spot measurements prior to end of shade period: mean ± sd, 93 ± 1.3 $\mu$mol mol$^{-1}$, $N = 328$ inductions), reference [CO$_2$] was controlled at 100 $\mu$mol mol$^{-1}$ during the shade. At the end of 30 min shade, PPFD was returned to its initial value of 1500 $\mu$mol m$^{-2}$ s$^{-1}$ and [CO$_2$] was set to the first of a stratified random sequence of ten [CO$_2$]s, measured at two min intervals so that chamber stability and IRGA matching could be achieved reliably. For each leaf to be measured, an independent sequence of reference [CO$_2$]s was drawn from the following set: 50, 100, 200, 300, 400, 500, 600, 700, 800, and 1000 $\mu$mol mol$^{-1}$. The [CO$_2$]s were ordered so that concentrations from the $\leq 400$ $\mu$mol mol$^{-1}$ and $\geq 500$ $\mu$mol mol$^{-1}$ ranges were interspersed randomly (e.g., 800, 200, 600, 100, 500, 400, 700, 300, 1000, 50), and were rotated over ten separate inductions so that every [CO$_2$] was measured at every interval between 2 and 20 min following shade (Supplementary Fig. 2). To aid with consistency of responses, measurements were made in the laboratory (i.e., low light, and relatively constant temperature and humidity conditions), and between inductions gas exchange was allowed to fully recover to steady state at reference [CO$_2$] of 430 $\mu$mol mol$^{-1}$. To ensure that induction measurements for a leaf could be captured within a single day, two LI-6800F were used, attached adjacent to one another, either side of the mid-rib.

Models

The relationship between $A$ and incident PPFD was modelled as a non-rectangular hyperbola (Long & Hallgren 1985):

$$A = \frac{\phi I + A_{sat}}{20} - \frac{\sqrt{(\phi I + A_{sat})^2 - 4\phi IA_{sat} - R_d}}{20}$$
Where: $\phi$ is the apparent quantum yield (mol mol$^{-1}$); $I$, incident PPFD ($\mu$mol m$^{-2}$ s$^{-1}$); $A_{\text{sat}}$, the maximum gross rate of leaf CO$_2$ assimilation ($\mu$mol m$^{-2}$ s$^{-1}$); $\theta$, a dimensionless curvature parameter; and $R_d$, day respiration ($\mu$mol m$^{-2}$ s$^{-1}$).

With values for [CO$_2$] in partial pressure units, the FvCB model (von Caemmerer & Farquhar 1981; Farquhar et al. 1980) was used to characterise $A/c_i$ relationships:

$$A = \min(W_C, W_I, W_P) \left( 1 - \Gamma^*/c_i \right) - R_d$$

$$W_C = V_{\text{c, max}} c_c / (c_c + K_{\text{CO}})$$

$$W_I = I c_c / (4c_c + 8\Gamma^*)$$

$$W_P = 3 T_P c_c / (c_c - \Gamma^*)$$

where $W_C$ is the Rubisco limited, $W_I$ electron transport limited, and $W_P$ triose-phosphate utilisation limited rate of carboxylation. The [CO$_2$] at the site of carboxylation in the chloroplast, $c_c = c_i - A/g_m$. Additional parameters are: $\Gamma^*$, the photosynthetic CO$_2$ compensation point in the absence of $R_d$; $V_{\text{c, max}}$, the maximum carboxylation rate of Rubisco; $K_{\text{CO}} = K_c (1 + O/K_O)$, where $K_c$ and $K_O$ are the respective Michaelis constants for Rubisco catalysis of carboxylation and oxygenation reactions, and $O$ is the partial pressure of O$_2$; $J$, electron transport rate; $T_P$, the rate of triose phosphate utilisation.

To identify the match between $c_i$ and $W_C$, $W_I$, and $W_P$ as limiting factors we used the approach of Gu, Pallardy, Tu, Law & Wullschleger (2010), fitting values for $V_{\text{c, max}}$, $J$, and $T_P$ using:

$$A = \frac{b - \sqrt{b^2 - 4c}}{2}$$

For $A_C$:

$$b = V_{\text{c, max}} - R_d + g_m (c_i + K_{\text{CO}})$$

$$c = g_m \left( V_{\text{c, max}} (c_i - \Gamma^*) - R_d (c_i + K_{\text{CO}}) \right)$$

For $A_J$.
For each $A/c_i$ response, all possible limitation-state combinations were tested, given the required order of limitation states along the $c_i$ axis ($W_C < W_J < W_P$), and the minimum number of data necessary for each limitation state ($N \geq 2$ when $K_{CO}$ and $I^*$ are fixed). The R Language and Environment function `optim` (R Core Team 2018) was used to minimise the distribution-wise cost function, accepting the model with the lowest value after checking for admissibility and testing for co-limited ‘swinging points’ (Gu et al. 2010).

Using this method, estimation of $g_m$ from the data was found not to credibly predict limitation states indicated by $\Phi_{PSII}$ (e.g., Busch & Sage 2017), so for consistency $g_m$ was assumed to be infinite throughout (approximated by setting $g_m$ to $1 \times 10^6 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$).

Values for $V_{c,max}$, $J$ and $T_P$ are thus apparent rates, and in the dynamic $A/c_i$ analysis are confounded with any dynamic variation in $g_m$. Similarly, to ensure credible values, mean leaf temperatures measured in the LI-6800F were used to predict $I^*$, $K_C$ and $K_O$, using values for tobacco (Sharkey, Bernacchi, Farquhar & Singsaas 2007). Combining the Sharkey et al. (2007) coefficients with estimation of $R_d$ as part of the fitting process provided the best fit in the region around $I^*$ for parameterisation of steady-state responses (for comparisons among parameterisations, see Supplementary Fig. 3).

In the dynamic $A/c_i$ analysis, where greater measurement error and a slightly reduced number of measurements made least-squares fits less reliable, genotype-level parameters from the steady-state $A/c_i$ measurements were used to ensure $A/c_i$ fits provided a reasonably close match with limitation states indicated by $\Phi_{PSII}$ (Supplementary Fig. 2). The value of $R_d$
was fixed. In addition, \( A_p \) was initially assigned only to points with \( c_i \geq \) that at which limitation transitioned from \( J \) to \( T_p \) in the steady-state. If best-fit, admissible models predicted \( T_p \), they were only accepted if they also predicted \( V_{c,\text{max}} \) and \( J \), otherwise data assigned to \( A_p \) were dropped and the model was refit, dropping the highest \( c_i \) data as necessary until a best-fit admissible model was found that either (a) included both \( A_C \) and \( A_I \), or (b) included \( A_C \) alone. When a best fit model with \( A_C \) alone was reached, because identification of \( A_I \) requires \( N \geq 2 \), the uppermost \( c_i \) value was dropped to prevent mis-attribution of data that could be assigned to \( A_I \) and the model was refit, taking the highest \( c_i \) used as a lower-bound value for \( c_{i,\text{trans}} \).

Stomatal limitation (\( L_S \)) was calculated from the steady-state \( A/c_i \) responses following Farquhar & Sharkey (1982):

\[
L_S = \frac{A_0 - A}{A_0}
\]

Where, \( A_0 \) is a reference net CO\(_2\) assimilation rate predicted at a \( c_i \) equal to leaf external [CO\(_2\)], and \( A \) was the rate observed at the initial reference [CO\(_2\)] of 430 μmol mol\(^{-1}\).

**Analyses of Rubisco activity, and content of Rubisco, total soluble protein, and chlorophylls**

Leaf samples consisting of four leaf discs (2.2 cm\(^2\) per sample) were homogenised in 0.6 mL of extraction buffer (50 mM Bicine-NaOH pH 8.2, 20 mM MgCl\(_2\), 1 mM EDTA, 2 mM benzamidine, 5 mM \( \varepsilon \)-aminocaproic acid, 50 mM 2-mercaptoethanol, 10 mM dithiothreitol, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, Mo, USA), and 1 mM phenylmethylsulphonyl fluoride) using an ice-cold mortar and pestle. Rapid grinding (< 60 s) was followed by centrifugation of the homogenate at 4 °C, 21000 g for 1 min. The supernatant was collected and used to determine Rubisco total activity by \(^{14}\)CO\(_2\) incorporation into acid-stable products as described previously (Carmo-Silva et al. 2017). The supernatant (20 mm\(^3\)) was incubated for 3 min in 500 mm\(^3\) of reaction mixture (100 mM
Bicine-NaOH pH 8.2, 20 mM MgCl\textsubscript{2}, 10 mM NaH\textsuperscript{14}CO\textsubscript{2} [9.25 kBq µmol\textsuperscript{-1}], and 2 mM KH\textsubscript{2}PO\textsubscript{4}) to fully carbamylate Rubisco. RuBP was then added (to 0.6 mM) to initiate the reaction, and assays quenched with 10 M formic acid after 30 s. Reaction mixtures were dried, the residue re-suspended, and scintillation counted as described previously (De Souza, et al. 2020). The same supernatant was used to determine Rubisco content by mixing 100 mm\textsuperscript{3} of supernatant with 100 mm\textsuperscript{3} of CABP binding buffer (100 mM Bicine-NaOH pH 8.2, 20 mM MgCl\textsubscript{2}, 20 mM NaHCO\textsubscript{3}, 1.2 mM \textsuperscript{14}C]CABP [carboxyarabinitol-1,5-bisphosphate, 37 kBq µmol\textsuperscript{-1}]), incubating at ~ 20 °C for 30 min, then following the column-based \textsuperscript{14}C]CABP binding assay described previously (Sharwood, Sonawane, Ghannoum & Whitney 2016).

Total soluble protein (TSP) was determined for aliquots taken from the supernatant used for Rubisco analyses via Bradford assay (Bradford 1976). Chlorophyll content was determined from an aliquot of the leaf homogenates prior to centrifugation, which was added to ethanol (Wintermans & de Mots 1965). Absorbance for TSP and chlorophyll determinations was measured in a SPECTROstar Nano microplate reader (BMG LabTech, Aylesbury, UK).

**Statistical analyses**

Modelling and statistical analyses were carried out using R Language and Environment 3.5.2 (R Core Team 2018). Among species differences were tested using one-way anova and Tukey’s Honest Significant Difference, and the homogeneity assumption was validated using Bartlett’s test.

For parameters from dynamic $A/c_i$ analysis, generalised additive mixed models (GAMM, package mgcv version 1.8-26) were used to summarize time-dependent changes without the need to assume particular underlying mechanisms. When fitting GAMM,
Brassica species were treated as fixed effects, allowing unique species-level functions with respect to time. Independently measured plants were treated as random effects influencing variance around the species-level functions (Zuur, Ieno, Walker, Saveliev & G M Smith 2009). The slopes of fitted functions for $V_{c,\text{max}}$ against time ($dV_{c,\text{max}}/dt$) from dynamic $A/c_i$ were obtained by finite differencing from values predicted by GAMM at 1 s resolution. Species specific confidence intervals for GAMM were approximated as: predicted values ± $t_{1-\alpha, \text{edf}} \times \text{SEM}$, where $\alpha = 0.025$, and edf = estimated degrees of freedom at the species level.

Results

Steady state photosynthesis and biochemical characteristics

Photosynthetic response to light and leaf biochemistry

Leaf level responses to PPFD (Fig. 1) showed mean values of $A_{\text{sat}}$, $R_d$, and $\theta$ that were highest for B. rapa, slightly lower for B. napus, and lowest for B. oleracea (Fig. 1). By contrast, $\phi$ was greater in B. oleracea and B. napus than in B. rapa. There was limited support for significant differences in $R_d$ ($F_{2,9} = 2.22$, $P = 0.16$) and $\phi$ ($F_{2,9} = 2.56$, $P = 0.13$) across the three Brassica. However, differences in $A_{\text{sat}}$ were marginally significant ($F_{2,9} = 3.03$, $P = 0.099$), and there was strong evidence for a significant difference in $\theta$ ($F_{2,9} = 9.91$, $P = 0.005$).

The smaller $\theta$ for B. oleracea compared with B. napus and B. rapa, supports a more gradual transition from light- to carboxylation-limited photosynthesis at higher PPFDs and was significant for both individual comparisons ($P \leq 0.026$).

The observed patterns of differences in mean Rubisco total activity and Rubisco amount were consistent with marginally significant differences in mean $A_{\text{sat}}$. Rubisco amount and total activity were lower in B. oleracea than in B. napus and B. rapa (Table 1), though these differences were not significant among the three species ($F_{2,12} \leq 1.6$, $P \geq 0.24$). Normalised to Rubisco content, Rubisco specific activities were even more similar than total
activities among the three Brassica (Table 1), implying that patterns of difference in total activity were strongly affected by amounts of Rubisco protein per unit leaf area. Interestingly, while the lower Rubisco content of B. oleracea leaves was paired with similar total soluble protein to B. rapa (P = 0.94), these two species showed marked differences in chlorophylls. B. oleracea had approximately double the amount of chlorophyll a+b (P < 0.001), and lower chlorophyll a:b ratios (P = 0.001) compared with B. rapa (Table 1). By contrast, B. napus had higher soluble protein content compared with the other two Brassica (P ≤ 0.029; Table 1), intermediate chlorophyll content (B. napus-B. oleracea, P = 0.084; B. napus-B. rapa, P = 0.002) and intermediate chlorophyll a:b ratio (B. napus-B. oleracea, P = 0.089; B. napus-B. rapa, P = 0.089). Thus, while Rubisco content was aligned with A_sat, it was opposite to investments in chlorophyll pigments, which were significantly less in leaves of B. rapa compared with B. oleracea.

Photosynthetic response to CO2

Operating point A and g_sw were significantly lower for B. oleracea than for B. rapa (A, P = 0.021; g_sw, P = 0.017). For both A and g_sw, B. napus was intermediate between the other Brassica: there was a marginally significant difference in A between B. napus and B. oleracea (P = 0.064); little support for a significant difference in g_sw between them (P = 0.15); and no significant difference in either A or g_sw between B. napus and B. rapa (P ≥ 0.31; Table 2). The significant differences between A and g_sw of B. oleracea and B. rapa were associated with an increase in mean c_i from 26.5 (B. oleracea) to 29.3 Pa (B. rapa), but measurements were not sufficiently repeatable across the small number of replicates to establish a significant difference in c_i among the three species (F_2,9 = 2.56, P = 0.13; Table 1).

The similarity in operating c_i, and differences in A and g_sw between the Brassica were associated with differences in steady state A/c_i responses (Fig. 2; Supplementary Fig. 1).
Mean $V_{c,max}$ and $J$ were, as for $A$, highest in $B. rapa$, intermediate in $B. napus$, and lowest in $B. oleracea$. While the three primary rate limiting factors: $V_{c,max}$, $J$ and $T_p$, were not significantly different between the three Brassica ($F_{2,9} \leq 2.16, P \geq 0.17$; Fig. 2), differences in $L_S$ were ($F_{2,9} = 5.01, P = 0.035$), specifically between $B. rapa$ and $B. oleracea$ ($P = 0.037$, other comparisons $P \geq 0.089$; Table 2). There was also a marginally significant difference in $c_{i,trans}$ ($F_{2,9} = 4.1, P = 0.054$), with $B. oleracea$ showing the highest $c_{i,trans}$ and $B. rapa$ the lowest: the range of $c_i$ that is expected to result in $V_{c,max}$ limiting $A$ was significantly greater for $B. oleracea$ than $B. rapa$. In combination, small differences in $V_{c,max}$, $J$, and $g_s$ led to operating $c_i$ that was significantly lower than $c_{i,trans}$ in $B. oleracea$ (one tailed, paired t-test: $t_3 = 3.61, P = 0.005$), but overlapped with $c_{i,trans}$ in $B. napus$ and $B. rapa$ (two tailed, paired t-test: $t_3 < \pm 1.69, P \geq 0.19$). Thus, in the steady state, carboxylation in leaves of $B. oleracea$ was limited by $V_{c,max}$, whereas $B. napus$ and $B. rapa$ operated at the transition between $V_{c,max}$ and $J$ limitation (Fig. 2; Supplementary Fig. 1). Finally, though at much higher $c_i$ than the operating point, a highly significant difference was also shown for the $c_i$ at which $A_J$ transitioned to $A_P$ ($F_{2,9} = 10.38, P = 0.006$), between $B. napus$, which had the lowest value for the $c_i$ of this transition, and $B. oleracea$, which had the highest (Fig. 2; $P = 0.005$).

**Photosynthetic induction**

*Recovery of $A$ during fast, mesophyll-dominated, and stomata-limited induction*

The vast majority of recovery in $A$ occurred while $c_i$ was decreasing, i.e., while recovery of $A$ was controlled primarily by non-stomatal factors (Fig. 3); recovery of $A$ during this 4-5 min period ($t_{ci,min}$, Table 3) averaged 77-84% ($R_{ci,min}$, Table 3). After 30 min shade at the relatively high shade-irradiance of 150 µmol m$^{-2}$ s$^{-1}$, ~70% of recovery occurred during the first 2 min (fast-phase), so slow-phase recovery prior to increases in $c_i$ accounted for ~10% of the shade-sun difference in $A$ (Table 3). When the fast- and slow-phase components of
non-stomatal-dominated recovery were taken together, neither their combined impact on recovery of $A$ nor their combined duration were significantly different between the three Brassica ($R_{c_{\text{c,min}}, P = 0.51; t_{c_{\text{c,min}}, P = 0.24}}$).

By contrast with non-stomatal-dominated induction, the remaining 20% of recovery in $A$, that was predominated by the effect of increasing $g_s$ on $c_i$, took significantly longer in $B$. oleracea than in $B$. rapa ($t_{A,90} - t_{c_{\text{c,min}}, Table 3; P = 0.02}$, and was marginally significantly longer in $B$. oleracea than $B$. napus (Tukey HSD, $P = 0.055$; Table 3). Mean $A$, $g_{\text{sw}}$ and $c_i$ of $B$. oleracea had not approached their steady-state values even after 20 min of induction (Fig. 4a), such that $t_{A,90}$ was significantly longer in $B$. oleracea than the other two species (Table 3; $F_2,9 = 7.24, P = 0.013; B$. oleracea-$B$.napus, $P = 0.034; B$. oleracea-$B$. rapa, $P = 0.017$).

Contrasting with $B$. oleracea, both $B$. napus and $B$. rapa reached $t_{A,90}$ within 10 min induction (Table 3), even though, like $B$. oleracea, their $g_{\text{sw}}$ and $c_i$ continued to increase beyond 20 min, $A$ was insensitive to this (Fig. 3 and 5).

**Apparent limiting biochemical factors during induction - dynamic $A/c_i$**

Progressive changes in $V_{c,\text{max}}$ determined from dynamic $A/c_i$ responses were qualitatively different between the three Brassica (Fig. 4). Increases in $V_{c,\text{max}}$ during induction were: 23% in $B$. oleracea, 33% in $B$.napus and 29% in $B$. rapa. The rate of change in $V_{c,\text{max}}$ ($dV_{c,\text{max}}/dt$) declined smoothly (Fig. 4d), and confirmed that increases in $V_{c,\text{max}}$ were predominantly over the first ~10 min of induction in $B$. oleracea, ~12 min in $B$. rapa (Fig. 4a, c & d), and ~18 min in $B$. napus (Fig. 4b & d). In all three, $V_{c,\text{max}}$ increased rapidly for the first 4-5 min of induction, coinciding with the $t_{c_{\text{c,min}}}$ observed in induction measurements (Table 3). It was also notable that $V_{c,\text{max}}$ of $B$. oleracea saturated before $t_{A,90}$ from the ambient induction experiments, whereas increases in $V_{c,\text{max}}$ of $B$. napus and $B$. rapa were continuing at their $t_{A,90}$, but with little subsequent effect on $A$ (Fig. 4).
The $c_i$ at which limitation transitioned away from $V_{c,\text{max}}$ ($c_{i,\text{trans}}$), which is co-determined by $V_{c,\text{max}}$ and $J$, was initially similar to ambient [CO$_2$] and decreased during induction. After 4-6 min induction, $c_{i,\text{trans}}$ was indistinguishable from steady-state values on the basis of approximate 95% confidence intervals (Fig. 5). Comparing time series for $c_{i,\text{trans}}$ (shade PPFD, 100 μmol mol$^{-1}$) with $c_i$ during induction at ambient [CO$_2$] (shade PPFD 150 μmol m$^{-2}$ s$^{-1}$; Fig. 5), by 20 min their values were essentially the same as those found at steady-state (i.e. B. oleracea, $c_i < c_{i,\text{trans}}$; B. napus, $c_i \sim c_{i,\text{trans}}$; B. rapa $c_i \sim c_{i,\text{trans}}$). Based on 95% confidence intervals, $c_i$ was significantly less than $c_{i,\text{trans}}$ throughout induction for B. oleracea (Fig. 5a), until ~ 10 min for B. napus (Fig. 5b), and until ~ 7 min in B. rapa (Fig. 5c), with $c_i$ intersecting mean $c_{i,\text{trans}}$ after 10-15 min induction in B. napus and B. rapa. Because $c_i < c_{i,\text{trans}}$ infers that $A$ is limited by $V_{c,\text{max}}$, as $c_i < c_{i,\text{trans}}$ throughout induction $A$ of B. oleracea was always $V_{c,\text{max}}$-limited, and the other two species were $V_{c,\text{max}}$ limited beyond $t_{A,90}$ (Table 3). Because $c_{i,\text{trans}}$ denotes a change in the slope of the $A/c_i$ response, overlap between $c_{i,\text{trans}}$ and $c_i$ of B. napus and B. rapa during induction explains why $A$ saturated while their $g_s$ and $c_i$ continued to increase (Fig 3b & c).

**Discussion**

Photosynthesis differed in several ways between B. rapa and B. oleracea. Most notably, the former had greater rates of gas exchange and recovered steady-state $A$ more rapidly following shade. B. napus was intermediate in most respects, although more similar to B. rapa. A novel dynamic $A/c_i$ response protocol that added randomisation of [CO$_2$]s during induction to a previous innovation of fixed low [CO$_2$] during shade (Taylor & Long 2017), imposed robust control for [CO$_2$] during induction. The dynamic $A/c_i$ experiments demonstrated that all three Brassica were limited by apparent $V_{c,\text{max}}$ for 10 min or more following 30 min shade. Importantly though, while B. oleracea stayed $V_{c,\text{max}}$ limited, B. napus and B. rapa transitioned
to co-limitation by $J$ after ~ 10 min. The transitions to co-limitation coincided broadly with saturation of $A$, explaining why ongoing increases in $g_s$ and/or $V_{c,max}$ had little subsequent effect in these two species, and providing a potential mechanistic explanation for previous observations of diversity among species in rates of recovery of $A$ relative to $g_s$ (Deans, Brodribb, Busch & Farquhar 2019b; McAusland et al. 2016).

*Limitations affecting steady-state photosynthesis*

The difference in limitation-states affecting steady-state $A$ of the three species was not an anticipated outcome, but was clear. All three operated within 5 Pa of their $c_{i,trans}$. This is consistent with the hypothesis that operation close to $c_{i,trans}$ reflects optimisation of resource investment between capacities for carboxylation and RuBP regeneration (von Caemmerer & Farquhar 1981; Farquhar & Sharkey 1982), and perhaps indicative of acclimation to recent rapid increases in atmospheric [CO$_2$] (Long et al. 2004; Kromdijk & Long 2016).

The amount of Rubisco and its total activity were a match for species differences in apparent $V_{c,max}$ and a better explanation of $V_{cmax}$ than differences in Rubisco performance. All three *Brassica* had similar Rubisco specific activities. Compared with Rubisco properties, differences in chlorophyll and total soluble protein were more easily detected. *B. oleracea* had double the chlorophyll a+b content compared with *B. rapa*, and the leaves of *B. oleracea* showed a more gradual transition away from light limitation as PPFD increased (significantly lower $\theta$). *B. oleracea* A12DHd had particularly thick, noticeably waxy leaves and may experience limited light saturation deeper in the mesophyll (Hikosaka & Terashima 1995), especially when using the red/blue light source of the LI-6800F (Terashima et al. 2009).

Reflectance from the waxy leaf surface may also reduce absorption by *B. oleracea* leaves and the species had lower chlorophyll a:b indicating a greater proportion of light harvesting chlorophylls, consistent with shade adaptation within the leaf. Evidence from biochemistry...
and light response curves is therefore consistent with linkages between different steady-state photosynthetic limitations in these *Brassica* and higher-level structural differences.

Limitation of \( A \) by apparent \( V_{c,\text{max}} \) in *B. oleracea* was clearly linked with lower \( g_s \) and greater \( L_S \) than in *B. napus* and *B. rapa*. The other key component of diffusive limitation affecting photosynthesis, \( g_m \), was not reliably estimated with our data using exhaustive dual optimisation. To obtain consistent visual matching between predicted limitation states and the inflexion of both \( A/c_i \) and \( \Phi_{\text{PSII}} \) in our three-species dataset required an effectively infinite value for \( g_m \). However, the modified exhaustive dual optimisation approach (Gu et al. 2010) is a powerful tool for identifying \( c_{i,\text{trans}} \) based on the inflexion of the \( A/c_i \) response, and incorporating a finite value for \( g_m \) in the model of photosynthesis does not affect whether operating point \( A \) falls above or below this inflexion.

Adequate fits for \( A/c_i \) responses in the region of \( \Gamma^* \) were achieved using the tobacco-derived parameterisation of Sharkey et al. (2007). By contrast, estimates of Rubisco kinetic parameters for *B. oleracea* reported in the literature (Hermida-Carrera, Kapralov & Galmés 2016) provided poor fits in this region (Supplementary Fig. 3). Compared with coefficients based on gas exchange measurements using tobacco (Sharkey et al. 2007), values for *B. oleracea* determined using *in vitro* measurements (Hermida-Carrera et al. 2016) are 7.5 Pa less for \( K_{\text{CO}_2} \), and 0.8 Pa greater for \( \Gamma^* \). As a consequence, Rubisco kinetic properties from Hermida-Carrera et al. (2016) predicted \( V_{c,\text{max}} \) to be ~ 6\% greater; however, their \( \Gamma^* \) exceeded the CO\(_2\) compensation points we measured in all three *Brassica* (Supplementary Fig. 3). While the parameterisation we used for \( g_m \) means that the reported biochemical rates of \( V_{c,\text{max}} \) and \( J \) incorporate differences in mesophyll properties, the fact that total activity of Rubisco from leaf extracts scaled with values for \( V_{c,\text{max}} \) strongly corroborates the finding of lower \( V_{c,\text{max}} \) in *B. oleracea*. Irrespective of the differences between published kinetic coefficients, therefore, *B. oleracea* had lower \( V_{c,\text{max}} \) and was \( V_{c,\text{max}} \) limited over a greater
range of $c_i$ than the other two species. Increasing Rubisco activity (e.g., Salesse-Smith, Sharwood, Busch, Kromdijk, Bardal & Stern 2018; Yoon et al. 2020) could be particularly useful for improvement of photosynthesis in *B. oleracea*, assuming the genotype tested here is representative of the species.

*Components of recovery in $A$ during induction.*

In all three *Brassica*, in addition to 70% of recovery attributable to fast-phase RuBP regeneration, and prior to increases in $c_i$ and $A$ linked with increasing $g_s$, slow-phase induction was initially dominated by non-stomatal effects consistent with Rubisco activation, which accounted for at least 10% of recovery in $A$. This fairly small value probably arose because of the relatively high PPFD ($150 \mu$mol m$^{-2}$ s$^{-1}$) used during shade, and the fact that steady-state $g_s$ was obtained in saturating light prior to imposing shade, hence relatively high $g_s$ at the start of induction (Kirschbaum & Pearcy 1988). Use of relatively high shade PPFD and pre-acclimation to saturating light makes our measurements most relevant to midday photosynthesis in upper layers of crop canopies (Burgess et al. 2016; Townsend et al. 2018; Zhu et al. 2004). In situations where initial $V_{c,max}$ and/or $g_s$ are lower, e.g., deeper layers of crop canopies where sunlit periods are interspersed by longer shade periods or preceded by persistent low light, more extended and larger impacts of $V_{c,max}$ would be expected when leaves are sunlit (Morales et al. 2018). The relatively high PPFD used here during shade also ensured that stomata remained the predominant route of water loss throughout our experiments, decreasing the risk of errors in calculated $c_i$ (Hanson, Stutz & Boyer 2016) and enabling use of $c_i$ as a sensitive indicator of whether mesophyll or diffusive factors were the predominant control over $A$.

The initial decrease in $c_i$ always extended to ~ 4-5 min of induction, at least twice the 2 min assumed to mark the end of the RuBP-regeneration dominated fast-phase. The 2 min
upper limit for the fast-phase is taken from the literature (e.g., Sassenrath-Cole & Pearcy 1992), and was used because gas exchange system mixing times meant that fast-phase kinetics could not be directly parameterised. The inflection of $A$ indicating the end of the fast phase nonetheless tended to occur slightly before 2 min (e.g., Fig. 3), so the estimate of photosynthetic recovery driven by Rubisco activation, at 2-3 min duration and 10%, is conservative. Evidence that shade-induced Rubisco deactivation can limit midday photosynthesis in field crops is consistent with previous detailed measurements of apparent $V_{c,max}$ following sun-shade-sun transitions in wheat (Taylor & Long 2017; Salter et al. 2019), and experiments that manipulated $Rca$ in rice (Yamori et al. 2012).

Beginning after 4-5 min of induction, increasing $g_s$ outweighed non-stomatal components as a determinant of increasing $c_i$ and $A$. At this time $c_{i,trans}$ was very close to its steady-state value. Despite the similar timing of transitions to $g_s$-dominated induction, recovery in $A$ was less strongly and persistently affected by $g_s$ in $B. napus$ and $B. rapa$ than $B. oleracea$. This might suggest that the prediction of Morales et al. (2018), based on careful reconstruction of photosynthetic regulation in Arabidopsis, that persistent stomatal limitation should be observed during longer light flecks, is not general across close crop relatives. There is evidence for considerable variation among plants, including different functional types, in the extent of stomatal limitation during induction (Deans, Brodribb, Busch & Farquhar 2019b; McAusland et al. 2016). Intraspecific studies addressing crops have also confirmed that the importance of stomatal limitations during induction can differ between species: stomata have little apparent importance in determining genetic variation for induction in soybean or rice (Acevedo-Siaca et al. 2020; Soleh et al. 2016), but are a dominant factor in cassava (De Souza et al. 2020).
To evaluate dynamic changes in $V_{c,max}$ and Rubisco limitation in planta requires dynamic $A/c_i$ response measurements (Chazdon & Pearcy 1986; Salter et al., 2019; Soleh et al. 2016; Taylor & Long 2017). To avoid the potential caveat of [CO$_2$] effects on half times for photosynthetic induction (Kaiser et al. 2017; Woodrow et al. 1996), the new dynamic $A/c_i$ protocol used here varied [CO$_2$] during every induction. This increased the interval between measurements to 2 min compared with 10 s in previous studies (Salter et al. 2019; Soleh et al. 2016; Taylor & Long 2017), so half times for apparent $V_{c,max}$ based on exponential curve fitting (Salter et al. 2019; Taylor & Long 2017) were less reliable and we analysed time series using GAMM. Though more qualitative, this analysis provided evidence that increases in apparent $V_{c,max}$ of B. napus are sustained over longer periods than in the other two; it augmented the traditional perspective of a two-phase RuBP regeneration and Rubisco activation limited sequence (Pearcy et al. 1996) by providing evidence for transitions to co-limitation by $J$ after ~10 min of induction in B. napus and B. rapa; and it correctly reproduced limitation-states observed in steady-state measurements 20 min into induction.

As with induction experiments, recovery of apparent $V_{c,max}$ was evaluated following shade treatments consistent with expectations for field crops (Burgess et al. 2016; Townsend et al. 2018; Zhu, Ort, Whitmarsh & Long 2004). The relatively high PPFD used to simulate shade may explain the smaller increases in $V_{c,max}$ (23-33% compared with > ~40%) than were observed in sun-shade-sun experiments with wheat (Salter et al. 2019; Taylor & Long 2017). Timescales for increases in apparent $V_{c,max}$ were, however, consistent with those of wheat, i.e., saturating after 10-15 min induction. That apparent $V_{c,max}$ continued to increase after $t_{ci,min}$ agrees with results from both dynamic $A/c_i$ (Chazdon & Pearcy 1986) and $A^*$ ($c_i$-corrected $A$, Woodrow & Mott 1989) methods used to establish the duration and impacts of
slow-phase limitations. Our results therefore validate the use of those values to model impacts of Rubisco activation during induction (Morales et al. 2018; Wang et al. 2020).

As \( c_i \) increased during induction, after \( \sim 10 \) min it began to coincide with and exceed \( c_i,\text{trans} \) of both \( B. \text{napus} \) and \( B. \text{rapa} \). This experimental outcome has important consequences for both the simplified \( A^* \) approach to evaluation of biochemical limitations (Woodrow & Mott 1989; Hammond et al. 1998) and a recent method incorporating more detailed models of leaf gas exchange to quantify stomatal limitation based on more realistic assumptions about the shape of the \( A/c_i \) response (Deans et al. 2019a). Both methods assume Rubisco limitation, and our results suggest this is valid in broad terms, but the methods will suffer from reduced accuracy if and when \( c_i \) approaches \( c_i,\text{trans} \), because \( c_i,\text{trans} \) marks an inflection in the response of \( A \) to \( c_i \).

Persistent \( V_{c,\text{max}} \) limitation in \( B. \text{oleracea} \) meant that \( A \) continued to respond to changes in both \( V_{c,\text{max}} \) and \( g_s \) even after \( 20 \) min induction. By contrast, transitions to co-limitation by \( J \) after \( \sim 10 \) min induction in \( B. \text{napus} \) and \( B. \text{rapa} \), meant \( A \) subsequently showed decreased sensitivity to changing \( V_{c,\text{max}} \) and \( g_s \). \( B. \text{napus} \) and \( B. \text{rapa} \) therefore overcame the effects of shade on \( A \) more rapidly. Because \( c_i,\text{trans} \) marks an inflection in the response of \( A \) to \( g_s \), it has been argued that steady-state operating points in the vicinity of \( c_i,\text{trans} \) can encompass a wide range of values for the marginal cost of water use (\( \delta E/\delta A \); von Caemmerer & Farquhar 1981; Farquhar & Sharkey 1982), compatible with a range of alternative water use strategies (Cowan & Farquhar, 1977; Cowan, 1982). An alternative view might be that operation close to \( c_i,\text{trans} \), as observed for \( B. \text{napus} \) and \( B. \text{rapa} \), results in more rapid declines in \( A/g_{sw} \) (intrinsic water use efficiency) during induction, compared with \( c_i < c_i,\text{trans} \), i.e., persistent Rubisco limitation as in \( B. \text{oleracea} \). Do faster photosynthetic responses to shade among crop plants trade-off against regulation of leaf water status?

Further characterisation of the temporal characteristics and/or frequency of deviations
between $c_i$ and $c_{i,\text{trans}}$ using dynamic $A/c_i$ might provide useful insights into trade-offs between optimisation of radiation and water use efficiencies.

Conclusions

Measurements of three agriculturally important Brassica showed that in addition to classic fast RuBP regeneration and slow $V_{c,\text{max}}$ limited phases, transitions to co-limitation by $J$ affect the dynamics of photosynthesis following shade. In leaves where $c_i$ approached $c_{i,\text{trans}}$ more quickly during induction, subsequent photosynthesis was less sensitive to ongoing changes in $V_{c,\text{max}}$ and $g_s$. Diurnal productivity of C$_3$ crops with lower $c_{i,\text{trans}}$ would therefore be expected to be less sensitive to shade. Finally, although only one genotype of each crop was examined, these crops can be interbred, and the variation identified here shows scope for physiologically guided breeding to achieve improved photosynthetic efficiency.
Acknowledgements

This work was supported by Lancaster University, and by a subaward from the University of Illinois as part of the research project Realizing Increased Photosynthetic Efficiency (RIPE) that is funded by the Bill & Melinda Gates Foundation, Foundation for Food and Agriculture Research, and the U.K. Department for International Development under grant number OPP1172157. The authors wish to thank George Goodwin (Elsoms Seeds Ltd.) and Graham Teakle (Warwick Crop Centre) for providing seeds; Dr. Shaun Nielsen for discussions around R programming for model fitting; and two anonymous reviewers and Prof. A.P.M. Weber for constructive feedback that improved the manuscript.

Data availability statement

Data for leaf biochemistry, steady state responses to PPFD and CO₂, induction responses and dynamic $A/c_i$ responses, are available at https://doi.org/10.17635/lancaster/researchdata/378

Conflict of interest

The authors declare that they have no conflict of interest
References

Acevedo-Siaca L.G., Coe R., Wang Y., Kromdijk J., Quick W.P. & Long S.P. (2020) Variation in photosynthetic induction between rice accessions and its potential for improving productivity. *New Phytologist* https://doi.org/10.1111/nph.16454.

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248–254.

Burgess AJ., Retkute R., Preston S.P., Jensen O.E., Pound M.P., Pridmore T.P., & Murchie E.H. (2016) The 4-dimensional plant: Effects of wind-induced canopy movement on light fluctuations and photosynthesis. *Frontiers in Plant Science*, 7, 1–12.

von Caemmerer S. & Farquhar G.D. (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* 153, 376–387.

von Caemmerer S. & Edmondson D. (1986). Relationship between steady-state gas exchange, *in vivo* Ribulose Bisphosphate Carboxylase activity and some carbon reduction cycle intermediates in *Raphanus sativus*. *Australian Journal of Plant Physiology*, 13, 669-688.

Carmo-Silva A.E. & Salvucci M.E. (2013) The regulatory properties of Rubisco Activase differ among species and affect photosynthetic induction during light transitions. *Plant Physiology* 161, 1645–1655.

Carmo-Silva E., Andralojc P.J., Scales J.C., Driever S.M., Mead A., Lawson T., … Parry M.A.J. (2017) Phenotyping of field-grown wheat in the UK highlights contribution of light response of photosynthesis and flag leaf longevity to grain yield. *Journal of Experimental Botany* 68, 3473–3486.
Chazdon R.L. & Pearcy R.W. (1986) Photosynthetic responses to light variation in rainforest species I. Induction under constant and fluctuating light conditions. *Oecologia* **69**, 524–531.

Cowan I.R. (1982) Regulation of water use in relation to carbon gain in higher plants. In *Encyclopedia of Plant Physiology New Series Volume 12 B.* (eds O.L. Lange, P.S. Nobel, C.B. Osmond & H. Ziegler), pp. 589–614. Springer Verlag, Berlin, Heidelberg, New York.

Cowan I.R. & Farquhar G.D. (1977) Stomatal function in relation to leaf metabolism and environment. In *Symposia of the Society for Experimental Biology.* pp. 471–505.

Deans R.M., Farquhar G.D. & Busch F.A. (2019a) Estimating stomatal and biochemical limitations during photosynthetic induction. *Plant, Cell & Environment*, **42**, 3227-3240.

Deans R.M., Brodribb T.J., Busch F.A. & Farquhar G.D. (2019b) Plant water-use strategy mediates stomatal effects on the light induction of photosynthesis. *New Phytologist* **222**, 382–395.

Farquhar G.D., von Caemmerer S. & Berry J.A. (1980) A Biochemical Model of Photosynthetic CO$_2$ Assimilation in Leaves of C$_3$ Species. *Planta* **149**, 78–90.

Farquhar G.D. & Sharkey T.D. (1982) Stomatal Conductance and Photosynthesis. *Annual Review of Plant Physiology* **33**, 317–345.

Fowler D., Coyle M., Skiba U., Sutton M.A., Cape J.N, Reis S., … Voss M. (2013) The global nitrogen cycle in the twenty-first century. Philosophical Transactions of the Royal Society B **368**, 20130164.

Glowacka K., Kromdijk J., Kucera K., Xie J., Cavanagh A.P., Leonelli L., … Long S.P. (2018) Photosystem II Subunit S overexpression increases the efficiency of water use in a field-grown crop. *Nature Communications* **9**, article number 868.
Gu L., Pallardy S.G., Tu K., Law B.E. & Wullschleger S.D. (2010) Reliable estimation of biochemical parameters from C₃ leaf photosynthesis-intercellular carbon dioxide response curves. *Plant, Cell and Environment* **33**, 1852–1874.

Haddeland I., Heinke J., Biemans H., Eisner S., Florke M., Hanasaki N., … Wisser D. (2014) Global water resources affected by human interventions and climate change. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 3251-3256.

Hammond E.T., John Andrews T., Mott K.A. & Woodrow I.E. (1998) Regulation of Rubisco activation in antisense plants of tobacco containing reduced levels of Rubisco activase. *Plant Journal* **14**, 101–110.

Hanson D.T., Stutz S.S. & Boyer J.S. (2016). Why small fluxes matter: The case and approaches for improving measurements of photosynthesis and (photo)respiration. *Journal of Experimental Botany*, **67**, 3027–3039.

Hermida-Carrera, C., Kapralov, M. V, & Galmés, J. (2016). Rubisco catalytic properties and temperature response in crops. *Plant Physiology*, **171**(August), pp.01846.2016

Hikosaka K. & Terashima I. (1995) A model of the acclimation of photosynthesis in the leaves of C₃ plants to sun and shade with respect to nitrogen use. *Plant, Cell & Environment* **18**, 605–618.

Inaba R. & Nishio T. (2002) Phylogenetic analysis of Brassiceae based on the nucleotide sequences of the S-locus related gene, SLR1. *Theoretical and Applied Genetics* **105**, 1159–1165.

Indermühle A., Stocker T.F., Joos F., Fischer H., Smith H.J., Wahlen M., … Stauffer B. (1999) Holocene carbon-cycle dynamics based on CO₂ trapped in ice at Taylor Dome, Antarctica. *Nature* **398**, 121–126.
Kaiser E., Morales A., Harbinson J., Kromdijk J., Heuvelink E. & Marcelis L.F.M. (2015) Dynamic photosynthesis in different environmental conditions. *Journal of Experimental Botany* **66**, 2415–2426.

Kaiser E., Morales A., Harbinson J., Heuvelink E., Prinzenberg A.E. & Marcelis L.F.M. (2016) Metabolic and diffusional limitations of photosynthesis in fluctuating irradiance in *Arabidopsis thaliana*. *Scientific Reports* **6**, 31252.

Kaiser E., Kromdijk J., Harbinson J., Heuvelink E. & Marcelis L.F.M. (2017) Photosynthetic induction and its diffusional, carboxylation and electron transport processes as affected by CO$_2$ partial pressure, temperature, air humidity and blue irradiance. *Annals of Botany* **119**, 191–205.

Kirschbaum M.U.F. & Pearcy R.W. (1988) Gas exchange analysis of the relative importance of stomatal and biochemical factors in photosynthetic induction in *Alocasia macrorrhiza*. *Plant Physiology* **86**, 782–785.

Kromdijk J, Glowacka K, Leonelli L, Gabilly ST, Iwai M, Niyogi KK, Long SP (2016) Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. *Science* **354**, 857-861.

Kromdijk J. & Long S.P. (2016) One crop breeding cycle from starvation? How engineering crop photosynthesis for rising CO$_2$ and temperature could be one important route to alleviation. *Proceedings of the Royal Society B*, **283**, 20152578.

Larson G., Piperno D.R., Allaby R.G., Purugganan M.D., Andersson L., Arroyo-Kalin M., … Fuller D.Q. (2014) Current perspectives and the future of domestication studies. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 6139–6146.

Lawson T., & Vialet-Chabrand S. (2019). Speedy stomata, photosynthesis and plant water use efficiency. *New Phytologist*, **221**, 93–98.
Liu S., Liu Y., Yang X., Tong C., Edwards D., Parkin I.A.P., … Paterson A.H. (2014) The 
Brassica oleracea genome reveals the asymmetrical evolution of polyploid genomes. 
Nature Communications 5, 3980.

Long, S.P. & Hallgren, J.-E. (1993). Measurement of CO₂ assimilation by plants in the field 
and the laboratory. In Photosynthesis and Production in a Changing Environment: a 
field and laboratory manual (eds D.O. Hall, J.M.O. Scurlock, H.R. Bolhār-
Nordenkampf, R.C. Leegood & S.P. Long), pp. 62–94. Chapman & Hall, London, 
United Kingdom.

Long S.P., Ainsworth E.A., Rogers A. & Ort D.R. (2004) Rising atmospheric carbon dioxide: 
plants face the future. Annual Review of Plant Biology. 55, 591-628.

McAusland L., Vialet-Chabrand S., Davey P., Baker N.R., Brendel O. & Lawson T. (2016) 
Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use 
efficiency. New Phytologist 211, 1209–1220.

Morales A., Kaiser E., Yin X., Harbinson J., Molenaar J., Driever S.M. & Struik P.C. (2018) 
Dynamic modelling of limitations on improving leaf CO₂ assimilation under fluctuating 
irradiance. Plant Cell and Environment 41, 589–604.

Mott K.A. & Woodrow I.E. (2000) Modelling the role of Rubisco activase in limiting non-
steady-state photosynthesis. Journal of Experimental Botany 51, 399–406.

Ort D.R., Merchant S.S., Alric J., Barkan A., Blankenship R.E., Bock R., … Zhu X.G. (2015) 
Redesigning photosynthesis to sustainably meet global food and bioenergy demand. 
Proceedings of the National Academy of Sciences 112, 8529–8536.

Parkin I.A.P., Gulden S.M., Sharpe A.G., Lukens L., Trick M., Osborn T.C. & Lydiate, D.J. 
(2005). Segmental structure of the Brassica napus genome based on comparative 
analysis with Arabidopsis thaliana. Genetics 171, 765–781.
Pearcy R.W., Krall J.P. & Sassenrath-Cole G.F. (1996) Photosynthesis in fluctuating light environments. In Photosynthesis and the Environment. (ed N.R. Baker), pp. 321–346. Kluwer Academic Publishers, Netherlands.

R Core Team (2018) R: A language and environment for statistical computing.

Rakow G. (2004) Species Origin and Economic Importance of Brassica. In: Pua E.C., Douglas C.J. (eds) Brassica. Biotechnology in Agriculture and Forestry 54, 3-12

Rana D., Van Den Boogaart T., O’Neill C.M., Hynes L., Bent E., Macpherson L., … Bancroft I. (2004) Conservation of the microstructure of genome segments in Brassica napus and its diploid relatives. Plant Journal 40, 725–733.

Raschke K. (1975) Stomatal action. Annual Review of Plant Physiology 26, 309–340.

Sage R.F. (1995) Was low atmospheric CO$_2$ during the pleistocene a limiting factor for the origin of agriculture. Global Change Biology, 1, 93-106.

Salesse-Smith C.E., Sharwood R.E., Busch F.A., Kromdijk J., Bardal V. & Stern D.B. (2018) Overexpression of Rubisco subunits with RAF1 increases Rubisco content in maize. Nature Plants 4, 802-810.

Salter W.T., Merchant A.M., Richards R.A., Trethowan R. & Buckley T.N. (2019) Rate of photosynthetic induction in fluctuating light varies widely among genotypes of wheat. Journal of Experimental Botany 70, 2787–2796.

Sassenrath-Cole G.F. & Pearcy R.W. (1992) The role of ribulose-1,5-bisphosphate regeneration in the induction requirement of photosynthetic CO$_2$ exchange under transient light conditions. Plant Physiology 99, 227–234.

Sharwood R.E., Sonawane B.V., Ghannoum O. & Whitney S.M. (2016) Improved analysis of C$_4$ and C$_3$ photosynthesis via refined in vitro assays of their carbon fixation biochemistry. Journal of Experimental Botany 67, 3137–3148.
Sharkey T.D., Bernacchi C.J., Farquhar G.D. & Singsaas E.L. (2007) Fitting photosynthetic carbon dioxide response curves for C₃ leaves. *Plant, Cell and Environment* **30**, 1035-1040.

Soleh M.A., Tanaka Y., Kim S.Y., Huber S.C., Sakoda K. & Shiraiwa T. (2017) Identification of large variation in the photosynthetic induction response among 37 soybean [*Glycine max* (L.) Merr.] genotypes that is not correlated with steady-state photosynthetic capacity. *Photosynthesis Research* **131**, 305–315.

Soleh M.A., Tanaka Y., Nomoto Y., Iwahashi Y., Nakashima K., Fukuda Y., … Shiraiwa T. (2016) Factors underlying genotypic differences in the induction of photosynthesis in soybean [*Glycine max* (L.) Merr.]. *Plant, Cell & Environment* **39**, 685–693.

De Souza A.P., Wang Y., Orr D.J., Carmo-Silva E. & Long S.P. (2020) Photosynthesis across African cassava germplasm is limited by Rubisco and mesophyll conductance at steady state, but by stomatal conductance in fluctuating light. *New Phytologist* **225**, 2498-2512.

Stephenson P., Baker D., Girin T., Perez A., Amoah S., King G.J. & Østergaard L. (2010) A rich TILLING resource for studying gene function in *Brassica rapa*. *BMC Plant Biology* **10**, 1–10.

Tanaka Y., Adachi S. & Yamori W. (2019) Natural genetic variation of the photosynthetic induction response to fluctuating light environment. *Current Opinion in Plant Biology* **49**, 52–59.

Taylor S.H. & Long S.P. (2017) Slow induction of photosynthesis on shade-sun transitions in wheat may cost at least 21% of productivity. *Philosophical Transactions of the Royal Society B: Biological Sciences* **372**, 20160543.
Terashima I., Fujita T., Inoue T., Chow W.S. & Oguchi R. (2009) Green light drives leaf photosynthesis more efficiently than red light in strong white light: revisiting the enigmatic question of why leaves are green. *Plant & Cell Physiology* **50**, 684–697.

Tilman D., Balzer C., Hill J. & Befort B.L. (2011) Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 20260–20264.

Townsend A.J., Retkute R., Chinnathambi K., Randall J.W.P., Foulkes J., Carmo-Silva E. & Murchie E.H. (2018) Suboptimal acclimation of photosynthesis to light in wheat Canopies. *Plant Physiology* **176**, 1233–1246.

Wang Y., Burgess S.J., de Becker E., & Long S.P. (2020). Photosynthesis in the fleeting shadows: An overlooked opportunity for increasing crop productivity? *The Plant Journal* **101**, 874–884.

Wintermans J.F.G.M. & De Mots A. (1965) Spectrophotometric characteristics of chlorophylls a and b and their phenophytins in ethanol. *Biochimica et Biophysica Acta (BBA) - Biophysics including Photosynthesis* **109**, 448–453.

Woodrow I.E., Kelly M. & Mott K.A. (1996) Limitation of the rate of ribulosebisphosphate carboxylase activation by carbamylation and ribulosebisphosphate carboxylase activase activity: development and tests of a mechanistic model. *Australian Journal of Plant Physiology* **23**, 141-149.

Woodrow I.E. & Mott K.A. (1989) Rate limitation of non-steady-state photosynthesis by ribulose-1,5-bisphosphate carboxylase in spinach. *Australian Journal of Plant Physiology* **16**, 487–500.
Yamori W., Masumoto C., Fukayama H. & Makino A. (2012) Rubisco activase is a key regulator of non-steady-state photosynthesis at any leaf temperature and, to a lesser extent, of steady-state photosynthesis at high temperature. *The Plant Journal* **71**, 871–880.

Yoon D.-K., Ishiyama K., Suganami M., Tazoe Y., Watanabe M., Imaruoka S., … Makino A. (2020). Transgenic rice overproducing Rubisco exhibits increased yields with improved nitrogen-use efficiency in an experimental paddy field. *Nature Food*, **1**, 134–139.

Zhu X.G., Ort D.R., Whitmarsh J. & Long S.P. (2004) The slow reversibility of photosystem II thermal energy dissipation on transfer from high to low light may cause large losses in carbon gain by crop canopies: A theoretical analysis. *Journal of Experimental Botany* **55**, 1167–1175.

Zhu X.G., Long S.P. & Ort D.R. (2010) Improving photosynthetic efficiency for greater yield. *Annual Review of Plant Biology* **61**, 235–61.

Zuur A.F., Ieno E.N., Walker N.J., Saveliev A.A. & G M Smith (2009) *Mixed Effects Models and Extensions in Ecology with R*. (eds M. Gail, K. Krickeberg, J.M. Samet, A. Tsiatis & W Wong), Springer, New York.
Table 1 Rubisco amount, specific and total activity for three Brassica (mean ± SEM, N = 5).

| Species      | Rubisco total activity (µmol m\(^{-2}\) s\(^{-1}\)) | Rubisco amount (g m\(^{-2}\)) | Rubisco specific activity (µmol g\(^{-1}\) s\(^{-1}\)) | Total soluble protein (g m\(^{-2}\)) | Chlorophylls a and b (g m\(^{-2}\)) | Chlorophyll a:b |
|--------------|---------------------------------------------------|--------------------------------|-----------------------------------------------------|-------------------------------------|-----------------------------------|----------------|
| B. oleracea  | 38 ± 4.0                                          | 1.61 ± 0.322                   | 25.5 ± 2.37                                         | 3.88 ± 0.218\(a\)                   | 0.500 ± 0.025\(a\)               | 2.14 ± 0.051\(a\)               |
| B. napus     | 46 ± 3.6                                          | 1.79 ± 0.147                   | 26.1 ± 0.86                                         | 4.83 ± 0.266\(b\)                  | 0.428 ± 0.025\(a\)               | 2.28 ± 0.02\(ab\)               |
| B. rapa      | 48 ± 4.9                                          | 1.87 ± 0.23                    | 25.7 ± 0.62                                         | 3.77 ± 0.185\(b\)                  | 0.290 ± 0.014\(b\)               | 2.42 ± 0.049\(b\)               |

Different superscripts indicate significant differences at P < 0.05 using Tukey’s HSD.

Table 2 Steady-state values for leaf net CO\(_2\) assimilation (\(A\)), stomatal conductance to H\(_2\)O (\(g_{sw}\)), intrinsic water use efficiency (iWUE = \(A/g_{sw}\)), intercellular [CO\(_2\)] (\(c_i\)), \(c_i\) for the limitation-state transition from \(V_{c,max}\) to \(J (c_{i,trans})\), and stomatal limitation (\(L_S\)) of three Brassica, at: PPFD, 1500 µmol m\(^{-2}\) s\(^{-1}\); leaf temperature, 25 °C; and leaf-air vapour pressure deficit, 1.2 kPa, and CO\(_2\) ~ 400 µmol mol\(^{-1}\) (mean ± SEM, N = 4).

| Species      | \(A\) (µmol m\(^{-2}\) s\(^{-1}\)) | \(g_{sw}\) (mol m\(^{-2}\) s\(^{-1}\)) | \(c_i\) (Pa)  | \(c_{i,trans}\) (Pa) | \(L_S\) (%) |
|--------------|-----------------------------------|---------------------------------------|----------------|----------------------|-------------|
| B. oleracea  | 32.5 ± 0.69\(a\)                 | 0.46 ± 0.055\(a\)                    | 26.5 ± 0.95    | 35.1 ± 1.55\(a\)     | 21.9 ± 2.24\(a\)          |
| B. napus     | 36.6 ± 1.67\(ab\)               | 0.63 ± 0.040\(ab\)                   | 28.3 ± 0.40    | 31.6 ± 2.07\(ab\)    | 14.5 ± 1.98\(ab\)         |
| B. rapa      | 37.7 ± 0.44\(b\)                | 0.75 ± 0.071\(b\)                    | 29.3 ± 1.04    | 28.8 ± 0.7\(b\)      | 12.8 ± 2.21\(b\)          |

Different superscripts indicate significant differences at P < 0.05 using Tukey’s HSD.
Table 3 Statistical summary of photosynthetic induction characteristics in three *Brassica*.

Mean ± SEM (N = 3, *B. oleracea*; N = 4, *B. napus* & *B. rapa*).

| Species | *B. oleracea* | *B. napus* | *B. rapa* |
|---------|---------------|------------|-----------|
| Recovery in A at end of fast phase: two minutes after shade \( (R_{\text{fast}}, \%) \) | 64 ± 4.9 | 72 ± 4.8 | 72 ± 3.2 |
| Recovery in A, at \( c_i \) minimum \( (R_{\text{ci,min}}, \%) \) | 77 ± 5.0 | 81 ± 3.7 | 84 ± 3.5 |
| Slow phase recovery \( (R_{\text{ci,min}} - R_{\text{fast}}, \%) \) | 12.6 ± 0.77 | 10 ± 2.41 | 11.8 ± 1.81 |
| Time to \( c_i \) minimum* \( (t_{\text{ci,min}}, \text{min}) \) | 5.2 ± 0.59 | 4.1 ± 0.34 | 4.6 ± 0.34 |
| Time to 90% recovery of A \( (t_{A,90}, \text{min}) \) | 16.7 ± 3.49<sup>a</sup> | 8.7 ± 2.02<sup>b</sup> | 7.4 ± 1.41<sup>b</sup> |
| Duration of recovery associated with increasing \( c_i \) \( (t_{A,90} - t_{\text{ci,min}}, \text{min}) \) | 11.5 ± 3.28<sup>a</sup> | 4.6 ± 1.71<sup>ab</sup> | 2.75 ± 2.2<sup>b</sup> |

Different superscripts indicate differences with P < 0.1 using Tukey’s HSD.
**Figure Captions**

**Fig. 1** Responses of photosynthesis to light, for three *Brassica* species: (a) *B. oleracea*; (b) *B. napus*; (c) *B. rapa*. Non-rectangular hyperbola parameters: effective quantum yield ($\phi$), asymptotic gross CO$_2$ assimilation rate ($A_{sat}$), curvature ($\theta$), and day respiration ($R_d$) are provided as mean ± SEM (N=4) across models fit to independent replicates within each species. Lines represent combined parameter means, and two representative sets of data are shown.

**Fig. 2** CO$_2$ response curves show that shifts in operating $c_i$, and the $c_i$ at which the factor limiting net CO$_2$ assimilation rate transitions from $V_{c,max}$ to $J$, result in different biochemical limitations of steady state photosynthesis among three *Brassica* species: (a) *B. oleracea* (circles); (b) *B. napus* (diamonds); (c) *B. rapa* (triangles). Maximum net CO$_2$ assimilation rates attributable to carboxylation limited by Rubisco ($A_C$), electron transport ($A_J$), and triose phosphate utilisation ($A_P$); CO$_2$ compensation point ($\Gamma$); and $c_i$ values marking transitions between biochemical limiting factors, are plotted relative to mean operating points (grey fill, SEM smaller than symbol size). Also shown, are mean ± SEM (N=4) for maximum Rubisco limited carboxylation rate ($V_{c,max}$), electron transport rate ($J$), and triose phosphate utilisation ($T_P$). Shading distinguishes two example data sets per species. Models were fit to data for individual leaves before summarizing parameters.

**Fig. 3** Induction of net CO$_2$ assimilation ($A$), stomatal conductance ($g_{sw}$), and intercellular CO$_2$ ($c_i$) for three *Brassica* species, responding to an abrupt shift in photosynthetic photon flux density (PPFD), to 1500 µmol m$^{-2}$ s$^{-1}$ after 30 minutes at 150 µmol m$^{-2}$ s$^{-1}$. Mean ± SEM for (a) *B. oleracea* (N=3), (b) *B. napus* (N=4), (c) *B. rapa* (N=4). Dashed lines indicate steady state values obtained at 1500 µmol m$^{-2}$ s$^{-1}$ PPFD prior to shade.
**Fig. 4** Time dependence of $V_{c,max}$ (a-c) and $dV_{c,max}/dt$ (d) following induction for (a) *Brassica oleracea* (N = 4), (b) *B. napus* (N = 4), and (c) *B. rapa* (N = 3). Arrows indicate mean values for the time to recover 90% of $A$, measured in separate induction measurements at ambient $[\text{CO}_2]$ ($t_{A,90}$; Table 3).

**Fig. 5** Post-shade response of transition $c_i$ values ($c_{i,\text{trans}}$), at which biochemical limitation switches from maximum rate of carboxylation by Rubisco ($V_{c,max}$) to either rate of electron transport ($A_C/A_J$, open symbols) or triose phosphate limitation ($A_C/A_P$, closed symbols), and $c_i$ measured during induction at ambient $[\text{CO}_2]$ (small grey symbols, see also Fig. 3). Where $c_i < c_{i,\text{trans}}$ supports $V_{c,max}$ limitation, and $c_i > c_{i,\text{trans}}$ limitation by factors other than $V_{c,max}$. (a) *Brassica oleracea* (N = 4), (b) *B. napus* (N = 4), and (c) *B. rapa* (N = 3). Steady-state $c_{i,\text{trans}}$ (dashed lines; Table 2), and time to recover 90% of $A$, measured in separate induction measurements at ambient $[\text{CO}_2]$ (arrows, $t_{A,90}$; Table 3), are shown for reference.
Fig. 1

\begin{table}
\begin{tabular}{|c|c|c|}
\hline
 & \textbf{B. oleracea} & \textbf{B. napus} & \textbf{B. rapa} \\
\hline
\textbf{\(\phi\)} & 0.068 \pm 0.002 & 0.069 \pm 0.0019 & 0.064 \pm 0.0013 \\
\textbf{\(A_{\text{sat}}\)} & 40 \pm 1.66 & 43.9 \pm 1.68 & 44.9 \pm 0.95 \\
\textbf{\(\theta\)} & 0.69 \pm 0.031 & 0.79 \pm 0.018 & 0.83 \pm 0.017 \\
\textbf{\(R_d\)} & 2.2 \pm 0.145 & 2.69 \pm 0.118 & 2.78 \pm 0.308 \\
\hline
\end{tabular}
\end{table}
Fig. 2

(a) *B. oleracea*

\[
\begin{align*}
V_{c,\text{max}} &= 127 \pm 1.3 \\
J &= 221 \pm 5.2 \\
T_P &= 15.8 \pm 0.38
\end{align*}
\]

(b) *B. napus*

\[
\begin{align*}
V_{c,\text{max}} &= 138 \pm 9.7 \\
J &= 228 \pm 8.9 \\
T_P &= 15.7 \pm 0.54
\end{align*}
\]

(c) *B. rapa*

\[
\begin{align*}
V_{c,\text{max}} &= 145 \pm 4.6 \\
J &= 231 \pm 7.7 \\
T_P &= 16.3 \pm 0.82
\end{align*}
\]
Fig. 3

(a) *B. oleracea*

(b) *B. napus*

(c) *B. rapa*
Fig. 4

(a) *B. oleracea*  
(b) *B. napus*  
(c) *B. rapa*  
(d)  

\[ V_{\text{c,max}} \text{ (\text{\textmu{M}ol m}^{-2} \text{s}^{-1})} \]

\[ \frac{dV_{\text{c,max}}}{dt} \text{ (\text{\textmu{M}ol m}^{-2} \text{s}^{-1} \text{min}^{-1})} \]

\[ t_{A,90} \]

\[ 0 \quad 5 \quad 10 \quad 15 \quad 20 \quad 0 \quad 5 \quad 10 \quad 15 \quad 20 \]

\[ \text{time since shade (min)} \]
Fig. 5

(a) *B. oleracea*

(b) *B. napus*

(c) *B. rapa*

$c_i$ (Pa)

$t_{A,90}$

Time since shade (min)