RESEARCH PAPER

Relationship between irradiance and levels of Calvin–Benson cycle and other intermediates in the model eudicot Arabidopsis and the model monocot rice

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Abstract

Metabolite profiles provide a top-down overview of the balance between the reactions in a pathway. We compared Calvin–Benson cycle (CBC) intermediate profiles in different conditions in Arabidopsis (Arabidopsis thaliana) and rice (Oryza sativa) to learn which features of CBC regulation differ and which are shared between these model eudicot and monocot C₃ species. Principal component analysis revealed that CBC intermediate profiles follow different trajectories in Arabidopsis and rice as irradiance increases. The balance between subprocesses or reactions differed, with 3-phosphoglycerate reduction being favoured in Arabidopsis and ribulose 1,5-bisphosphate regeneration in rice, and sedoheptulose-1,7-bisphosphatase being favoured in Arabidopsis compared with fructose-1,6-bisphosphatase in rice. Photosynthesis rates rose in parallel with ribulose 1,5-bisphosphate levels in Arabidopsis, but not in rice. Nevertheless, some responses were shared between Arabidopsis and rice. Fructose 1,6-bisphosphate and sedoheptulose-1,7-bisphosphate were high or peaked at very low irradiance in both species. Incomplete activation of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase may prevent wasteful futile cycles in low irradiance. End-product synthesis is inhibited and high levels of CBC intermediates are maintained in low light or in low CO₂ in both species. This may improve photosynthetic efficiency in fluctuating irradiance, and facilitate rapid CBC flux to support photorespiration and energy dissipation in low CO₂.

Keywords: Arabidopsis, Calvin–Benson cycle, carbon dioxide, irradiance, metabolites, rice.

Introduction

Engineering photosynthesis is a promising route to improve crop yield (Long et al., 2015; Ort et al., 2015). In addition to the light reactions (Kromdijk et al., 2016) and photorespiration (Betti et al., 2016; South et al., 2019), there is increasing interest in optimizing the Calvin–Benson cycle (CBC) (Zhu et al., 2007; Driever et al., 2017; Simkin et al., 2017). Comprehensive...
information about metabolite levels will further these efforts by providing insights into the operation and regulation of the CBC, and by supporting the development and validation of metabolic models. The need for such information is emphasized by mounting evidence for interspecies variance in C₃ photosynthesis (Parry et al., 2008; Sage and Sage, 2009; Lawson et al., 2012; Galmés et al., 2014; Prins et al., 2016; Arrivault et al., 2019).

The CBC consists of three subprocesses; fixation of CO₂ by Rubisco to form two molecules of 3-phosphoglycerate (3PGA), reduction of 3PGA to triose phosphate (triose-P) using NADPH and ATP from the light reactions, and regeneration of ribulose 1,5-bisphosphate (RuBP) from triose-P (Heldt et al., 2005). Rubisco and three enzymes involved in RuBP regeneration [fructose-1,6-bisphosphatase (FBPase), sedoheptulose-1,7-bisphosphatase (SBPase), and phosphoribulokinase (PRK)] catalyse irreversible reactions, whilst the other reactions are near equilibrium (Basham and Krause, 1969; Stitt et al., 1980; Dietz and Heber, 1984; Mettler et al., 2014).

The CBC is regulated for two main reasons. First, to balance flux at the various enzymatic reactions and maintain metabolite levels in a range that allows optimal CBC operation (Lawson et al., 2012). Regulation must be rapid because environmental conditions, especially irradiance, can change rapidly, CBC fluxes are very high and metabolite pools are small, with half-times of <1 s (Stitt et al., 1980; Arrivault et al., 2009; Mettler et al., 2014). Secondly, unless they are inhibited by the order of 1000-fold, several CBC enzymes would catalyse wasteful futile cycles in the dark (Heldt et al., 2005).

Pioneering experimental and theoretical analyses emphasized that photosynthesis is restricted by the energy supply in light-limited conditions and by Rubisco in light-saturating conditions (Farquhar et al., 1980; von Caemmerer and Farquhar, 1981). In some conditions, such as low temperature, photosynthesis can be restricted by the rate of Pi recycling during end-product synthesis (Sharkey, 1985a, b; McClain and Sharkey, 2019). Subsequent work showed that photosynthesis can also be restricted by enzymes that convert triose-P to RuBP (Stitt and Sonnewald, 1995; Stitt, 1999; Stitt et al., 2010) including SBPase (Raines et al., 2000; Lefebvre et al., 2005; Zhu et al., 2007; Driever et al., 2017) and aldolase (Haake et al., 1998, 1999; Sunkin et al., 2017). The distribution of control in the CBC depends on pre-history and current conditions (Stitt and Schulze, 1994; Stitt et al., 2010), implying that optimal operation will require coordinated regulation at multiple sites.

A hierarchy of regulation mechanisms operating in different time scales and often affecting multiple enzymes regulates the CBC. Mass action rapidly propagates changes in metabolite levels through sequences of reactions that are close to thermodynamic equilibrium. For example, when a change in the supply of ATP or NADPH from the light reactions alters the 3PGA:triose-P ratio (Dietz and Heber, 1984), reversible reactions catalysed by triose phosphate isomerase, aldolase, transketolase, and phosphoriboseisomerase propagate the change in triose-P to fructose 1,6-bisphosphate (FBP), erythrose 4-phosphate (E4P), sedoheptulose 1,7-bisphosphate (SBP), sedoheptulose 7-phosphate (S7P), ribose 5-phosphate (R5P), xylulose 5-phosphate (Xu5P), and ribulose 5-phosphate (Ru5P). Product or allosteric regulation also mediate rapid responses (Stitt et al., 2010); for example, feedback inhibition of FBPase by fructose 6-phosphate (F6P) (Gardemann et al., 1986) and SBPase by S7P (Schimkat et al., 1990), and inhibition of PRK by RuBP, ADP, and 3PGA (Gardemann et al., 1983). The latter serves to prevent excess ATP consumption at the irreversible PRK reaction. Another fairly rapid response involves light-driven increases in stromal pH and Mg²⁺, which activate many CBC enzymes (Heldt et al., 2005). Slightly slower responses are facilitated by post-translational regulation, in particular thioredoxin-mediated redox regulation (Buchanan and Balmer, 2005). Thioredoxins activate many CBC enzymes including NADP-glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH), FBPase, SBPase, and PRK, as well as Rubisco activase (Laing et al., 1981; Scheibe, 1991; Buchanan and Balmer, 2005) and CP12, a small protein that interacts with and modulates NADP-GAPDH and PRK (Gontero and Maberly, 2012; Lopez-Calcagno et al., 2014). Rubisco is inhibited by binding of RuBP to the non-carbamylated form (Sharwood et al., 2016), by low molecular weight inhibitors that derive from catalytic infidelities of Rubisco, and by inhibitors such as 2-carboxy-d-arabinitol 1-phosphate that are synthesized by dedicated pathways (Yeoh et al., 1980; Moore et al., 1993; Parry et al., 2008). Release of these inhibitors and re-establishment of the active carbamylated form of Rubisco require dedicated enzymes such as Rubisco activase (Portis and Parry, 2007; Parry et al., 2008; Portis et al., 2008).

The CBC is autocatalytic, and pathway stoichiometry requires that, at steady state, five-sixths of the triose-P is retained in the CBC to regenerate RuBP. The remaining one-sixth is available for end-product synthesis. If triose-Ps are withdrawn too quickly, there will be a shortfall in RuBP, and if triose-Ps are withdrawn too slowly there will be a shortfall in inorganic phosphate (Pi) for ATP synthesis (Stitt, 1990; Stitt et al., 1987, 2010). CBC operation therefore depends on tight regulation of end-product synthesis. Cytosolic FBPase catalyses the first dedicated step in the conversion of triose-P to sucrose. This enzyme has strongly cooperative kinetics and is inhibited by the signal metabolite fructose 2,6-bisphosphate, whose level is regulated by 3PGA and Pi (Stitt, 1990). This regulatory network facilitates a threshold response, in which use of triose-P is inhibited until a threshold concentration is reached, and is strongly activated by further small increases in triose-P (Herzog et al., 1984; Stitt, 1990; Stitt et al., 1987). Starch synthesis is regulated via allosteric activation of ADP glucose pyrophosphorylase by a rising 3PGA/Pi ratio (Ballicora et al., 2004).

In addition to carboxylation of RuBP, Rubisco catalyses a side reaction with O₂ (Lorimer, 1981) leading to formation of 2-phosphoglycerolate (2PG) (Osmond, 1981; Sharkey, 1988; Betti et al., 2016; Hagemann et al., 2016). 2PG is salvaged via the photorespiratory pathway, in which two 2PG molecules are recycled to one 3PGA molecule with concomitant loss of CO₂. In current atmospheric conditions, about every fourth reaction of Rubisco leads to formation of 2PG, and this rises even further when stomata close during stress. Regulation is needed to maintain CBC flux and allow energy dissipation via photorespiration (Long et al., 2006; Arrivault et al., 2009; Eisenhut et al., 2017). For example, non-productive consumption of
RuBP in the Rubisco oxygenase reaction means that more than five-sixths of the triose-P must be retained in the CBC, and less than one-sixth is available for end-product synthesis. It is also becoming clear that photorespiratory intermediates influence CBC enzymes (Schimkat et al., 1990; Flügel et al., 2017).

It is not easy to predict how this hierarchy of regulation mechanisms interacts in vivo. Many CBC enzymes have highly cooperative kinetics and are influenced by multiple interacting effectors. For example, redox regulation often affects substrate affinity (see, for example, Gardemann et al., 1986), and substrate levels affect the midpoint redox potential of enzymes and hence the extent to which they are activated by thioredoxin (Ashton and Hatch, 1983; Scheibe, 1991; Ashton et al., 2000). These interactions allow >1000-fold changes in activity between light and darkness (Laing et al., 1981; Gardemann et al., 1983; Heldt et al., 2005). It can be envisaged that they facilitate coordinated regulation at multiple sites in the light, with fine modulation of enzymes allowing further fine tuning of flux in the CBC (Scheibe, 1991; Stitt, 1987; Fridlyand and Scheibe, 1999; Stitt et al., 2010). However, better understanding requires information about fluxes and metabolite levels in different conditions.

Full understanding of a metabolic network requires integration of knowledge into predictive models. Many CBC models are available with varying levels of complexity (see Arnold and Nikoloski, 2011, 2012). They range from simple but powerful abstractions based on Rubisco kinetics and pathway topology (Farquhar et al., 1980; von Caemmerer and Farquhar, 1981) to complex models utilizing detailed information about enzyme kinetic properties (Pettersson and Ryde-Pettersson, 1988; Fridlyand et al., 1999; Poolman, 2000; Laisk et al., 2006; Zhu et al., 2007). These models are only approximations due to our incomplete knowledge of the regulatory properties of enzymes, complex interactions between different mechanisms (see above), and subcellular compartmentation (Stitt et al., 1980; Gerhardt et al., 1987; Heldt et al., 2005). Computationally tractable models are likely to always require simplification, and their testing and validation require detailed information about metabolite levels in different conditions.

Information about the levels of CBC intermediates is surprisingly sparse. There were several studies of CBC intermediates under different conditions in the 1980s (Stitt et al., 1982a, 1983, 1984a, b; Badger et al., 1984; Dietz and Heber, 1984; Seemann and Sharkey, 1986, 1987; von Caemmerer and Edmondson, 1986; Sharkey and Seemann, 1989; Servais et al., 1989; summarized in Supplementary Table S1 at JXB online; for details, see Discussion). However, for technical and partly conceptual reasons, these early studies focused on RuBP and a handful of other intermediates (3PGA, triose-P, FBP, and F6P). In the last 10 years, new analytical platforms have been developed that combine chromatographic separation with tandem MS (LC-MS/MS), allowing near-comprehensive analysis of CBC intermediates (Cruz et al., 2008; Arrivault et al., 2009; Hasunuma et al., 2010; Ma et al., 2014). Arnold and Nikoloski (2012) found poor agreement between the metabolite levels predicted by various CBC models and those measured in Arabidopsis under ambient CO₂ and limiting irradiance (Arrivault et al., 2009). This underlines the need for a compendium of metabolite data sets. In particular, these new methods have not been systematically used to profile metabolites in different conditions, except for Arabidopsis at different CO₂ concentrations (Arrivault et al., 2009) and the model alga Chlamydomonas reinhardtii at different light intensities (Mettler et al., 2014).

It is also important to learn whether CBC operation is conserved across species. Since it originated ~2 billion years ago, the CBC has been subject to continued and powerful selection pressure because of changing atmospheric CO₂ and O₂ levels, as well as variation in temperature and the water and nutrient supply (Raven et al., 2017; Sage, 2017). There is considerable diversity in the rate of photosynthesis in C₃ species (Evans, 1989; Wullschleger, 1993), and mounting evidence for diversity in Rubisco kinetics (Galmés et al., 2014; Prins et al., 2016), how Rubisco is regulated (Parry et al., 2008), leaf anatomy, chloroplast location and density (Sage and Sage, 2009; Busch et al., 2013), and stomatal responses (Lawson et al., 2012) in C₃ species. Metabolite profiling provides an unbiased strategy to search for interspecies variance; changes in the balance between different enzymatic steps will lead to changes in the relative levels of pathway intermediates, irrespective of whether the underlying cause is changes in gene expression and protein abundance, enzyme kinetics, or regulatory networks. Arrivault et al. (2019) recently reported variation in CBC intermediate profiles between five C₃ species, including Arabidopsis and rice. However, a major challenge of interspecies studies is to establish whether the observed differences are due to species differences, environmental conditions, or complex genotype×environment interactions.

In this study, we have analysed the levels of CBC and related intermediates in Arabidopsis and rice at several light intensities, and in rice at two CO₂ concentrations. In combination with previously published data for the response to CO₂ in Arabidopsis (Arrivault et al., 2009), these data allow us to compare the response of the CBC to irradiance and CO₂ in a model eudicot and a model monocot C₃ species. The results confirm that there is interspecies variance in CBC operation. They also uncover conserved responses that may minimize energy loss in low irradiance and allow CBC flux to be maintained when photorespiration is rapid.

**Materials and methods**

**Chemicals**

N₂, O₂, and CO₂ were obtained from Air Liquide (Germany; https://industrie.airliquide.de), and chemicals from Sigma-Aldrich (Darmstadt, Germany; https://www.sigmaaldrich.com), Roche Applied Science (Mannheim, Germany; https://lifescience.roche.com), or Merck (www.merckmillipore.com).

**Plant growth and harvest**

Arabidopsis (Arabidopsis thaliana Col-0) was grown with an irradiance of 120 µmol m⁻² s⁻¹ until 35 d after sowing as in Arrivault et al. (2019). On the day of harvest, plants were illuminated at growth irradiance for 4–5 h and then were subjected to darkness, or illuminated at 20, 80, 280, or 440 µmol m⁻² s⁻¹ for 15 min, or left at 120 µmol m⁻² s⁻¹, before...
harvesting by cutting rosettes and quenching them instantaneously in a bath of liquid N2 under prevailing irradiance. Three rosettes were pooled per sample.

Rice (Oryza sativa ssp. indica cv. IR64) was grown at an irradiance of 350 μmol m−2 s−1 until 45-47 d after sowing as in Arrivault et al. (2019). Before harvest, plants were illuminated at growth irradiance for 4-8 h, adapted for 10 min under a mobile LED light array with dimmable LED lights (RHENAC Greentec AG, Hennef, Germany, www.rhenac-greentec.de) that were tuned to mimic the light spectrum within the growth chamber at 350 μmol m−2 s−1, and then illuminated at 50, 150, 250, 350, 450, 550, 650, or 730 μmol m−2 s−1 for 20 min. The first fully expanded leaf blades (two or three per sample) were harvested without shading by freezing clamp between two aluminium blocks that had been pre-cooled in liquid N2. Leaves were also harvested in darkness. In experiments where CO2 concentration was changed, 60-day-old rice plants were placed under the LED light array (350 μmol m−2 s−1) for 10 min and the first fully expanded leaf blades (two to three per sample) were then enclosed in a transparent gas-tight Plexiglas chamber (volume 68 ml), provided with 78% N2, 21% O2, and 500 ppm (ambient level) or 200 ppm CO2 (51 min−1) for 20 min, and quenched without shading by opening a small inlet and outlet and pouring liquid N2 into the chamber.

Metabolite analyses

Plant material was ground to a fine powder in a mortar pre-cooled in liquid N2 or in a cryo-robot (Stitt et al., 2007) and stored at −80 °C. Metabolites were extracted and quantified by LC-MS/MS using a reverse phase (Arrivault et al., 2009; all CBC metabolites, ADP-glucose (ADPG), UDP glucose (UDPG), glucose 1-phosphate (G1P), glucose 6-phosphate (G6P), or an anion exchange (Lunn et al., 1992; Merlo et al., 1993). Chl a and b were extracted and quantified as in Gibon et al., (2002) and protein as in Arrivault et al. (2019).

Gas exchange

CO2 assimilation was measured using whole rosettes of 5-week-old Arabidopsis or the mid-section of the first fully expanded leaves of 55- to 58-day-old rice plants using an open-flow infrared gas exchange analyser system (LI-6400XT; LI-COR Inc., Lincoln, NE, USA; www.lcor.com) equipped with an integrated fluorescence chamber head (LI-6400-17 whole-plant chamber for Arabidopsis; LI-6400-40, 2 cm2 leaf chamber for rice). CO2 was 400 μmol mol−1, leaf temperature 20 °C for Arabidopsis and 30 °C for rice, and relative humidity 65–75%.

Statistical analyses

Statistical analysis was performed in R Studio Version 1.1.463 (www.rstudio.com) with R version 3.5.1 (https://cran.r-project.org/) (details in figure legends and Supplementary Dataset S1).

Results

Light saturation response

Arabidopsis and rice were grown under limiting irradiance (120 μmol m−2 s−2 and 350 μmol m−2 s−2, respectively). Photosynthesis saturated at ~500 μmol m−2 s−1 irradiance with a light compensation point of 20±7 μmol m−2 s−1 in Arabidopsis, and ~2000 μmol m−2 s−1 with a light compensation point of 87±20 μmol m−2 s−1 in rice (Supplementary Fig. S1). The maximum rate of photosynthesis on a fresh weight basis was ~8-fold higher in rice than in Arabidopsis (1250 μmol CO2 g−1 FW h−1 and 145 μmol CO2 g−1 FW h−1, respectively). This partly reflects the higher protein and chlorophyll content in rice (2.95±0.32 mg chlorophyll g−1 FW, 71.6±12.2 mg protein g−1 FW) compared with Arabidopsis (1.14±0.12 mg chlorophyll g−1 FW, 32.2±2.6 mg protein g−1 FW). A relatively high light compensation point, light saturation response, and maximum rate of photosynthesis were previously reported for rice (Baker et al., 1990; Dingkuhn et al., 1990; Murchie et al., 1999; Sage and Sage, 2009).

Harvest and metabolite analyses

Arabidopsis was pre-illuminated at growth irradiance and then transferred for 15 min to a new irradiance (20, 80, 120, 280, and 440 μmol m−2 s−1) before harvesting whole rosettes. The chosen irradiances correspond, respectively, to the compensation point and ~67, 100, 230, and 360% of the irradiance required to half-saturate photosynthesis (Supplementary Fig. S1A). Rice was pre-illuminated at growth irradiance and then transferred for 20 min to a new irradiance (50, 150, 250, 350, 450, 550, 650, and 730, μmol m−2 s−1) before harvesting the first fully expanded leaf. The chosen irradiances correspond to the compensation point and ~30, 60, 100, 120, 150, and 170% of the irradiance that half-saturates photosynthesis (Supplementary Fig. S1B). The highest irradiance used for Arabidopsis and rice was ~90% and 75%, respectively, of that required to saturate photosynthesis. Metabolism was quenched instantaneously in Arabidopsis by submerging the rosettes in liquid N2 and in rice by clamping the leaf between two aluminium blocks pre-cooled in liquid N2. Quenching was performed at the prevailing irradiance. Samples were also collected in the dark.

Most intermediates of the CBC were quantified, including 3PGA, dihydroxyacetone phosphate (DHAP, also referred to as triose-P; glyceraldehyde 3-phosphate levels were below the detection limit and are probably 10–20 times lower than DHAP, due to the equilibrium constant of triose phosphate isomerase), FBP, F6P, SBP, 2PG, Ru5P, and RubP, as well as the photosynthetic intermediate 2PG. Ru5P and Xu5P were not resolved by LC-MS/MS and so were quantified together (termed ‘Ru5P+Xu5P’). Several intermediates of end-product synthesis were quantified including the starch synthesis intermediate ADPG, the sucrose synthesis intermediates G6P, G1P, UDPG, and Suc6P, as well as PEP and pyruvate. We assigned F6P to the CBC and G6P and G1P to sucrose synthesis because, due to plastid phosphoglucoisomerase being removed from equilibrium in the light, F6P is mainly located in the plastid and G6P and G1P are mainly in the cytosol (Dietz and Heber, 1984; Gerhardt and Heldt, 1984; Gerhardt et al., 1987; Szcadowska et al., 2013). Metabolite levels normalized on fresh weight and chlorophyll are provided in Supplementary Dataset S1. In addition, in a given sample, the amount of C within a given metabolite was divided by the total amount of C in all CBC intermediates plus 2PG. This transformation (termed ‘dimensionless’ or ‘normalized on total C’) removes effects due to a general change in metabolite levels and emphasizes changes in relative metabolite levels.
Principal component analysis of CBC intermediates

We first analysed the response of CBC intermediates and 2PG, starting with principal component (PC) analysis. PC analysis is a dimension-reduction technique that gives information about which samples (here, different species or irradiance) are closely related or separated, and which variables (here, metabolites) contribute to this relationship. The analysis was performed with z-scored data. The plots show the individual samples, and the mean value and 95% confidence limits for each species–irradiance treatment.

PC analysis on chlorophyll-normalized data (Fig. 1A) revealed a progressive shift of the metabolite profile in both species as irradiance increased. This was captured mainly in PC1, which represented 57% of total variance. Separation in PC1 was driven by almost all of the CBC intermediates, indicating that it is driven by a general increase in metabolite levels (see below for more data). Strikingly, Arabidopsis and rice showed different trajectories. This was captured in PC2, which accounted for 18% of total variance. Separation in PC2 was driven by FBP, F6P, and DHAP (higher in Arabidopsis; Fig. 2A–C), and SBP, Ru5P+Xu5P, 3PGA, and RuBP (higher in rice; Fig. 2A, B, D).

When PC analysis was performed on the dimensionless data sets (Fig. 1B), the response to increasing irradiance was weakened, especially for Arabidopsis. This normalization removes the effect of any general increase in metabolite levels (see above) allowing changes in relative levels to be seen more clearly. Arabidopsis and rice again showed different trajectories. This was captured in PC1, which represented 35% of the variance.

The analysis with the dimensionless data sets also highlighted that the lowest irradiance (20 µmol m⁻² s⁻¹ and 50 µmol m⁻² s⁻¹ in Arabidopsis and rice, respectively; in both cases, close to the light compensation point) separated strongly from other irradiances. This separation was mainly in PC2, which represented 23% of total variance, and was driven by high SBP and FBP (Fig. 2B). The low irradiance samples were also outliers when PC analysis was performed on the Arabidopsis samples only (Supplementary Fig. S3) or the rice samples only (Supplementary Fig. S4). The response was seen for both the chlorophyll-normalized and dimensionless data sets, and was driven by high SBP and FBP.

Responses of individual CBC intermediates

Responses of individual metabolites are shown in Fig. 2, using a shared y-axis scale to allow direct comparison of qualitative trends and absolute levels in the two species. To visualize the balance between different reactions, selected metabolite ratios were calculated (Fig. 3). The combined data set was analysed by one-way ANOVA to detect interspecies differences and differences within a species as irradiance increased (see Supplementary Dataset S1 for details). Responses to increasing irradiance in the individual species are highlighted in Supplementary Figs S2A and S5, which provide displays of metabolite levels and ratios using scales that optimally visualize the response in a given species and the results of one-way ANOVA performed separately for each species.

Visual inspection reveals some conserved responses between Arabidopsis and rice. First, many metabolites showed a progressive and significant increase as irradiance is increased, including 3PGA (except at the highest irradiance), DHAP, F6P, S7P, and 2PG in both species, and R5P, Ru5P+Xu5P, and RuBP across all light intensities in Arabidopsis and in the lower irradiance range in rice (Fig. 2; see also Supplementary Fig. S2A). Secondly, SBP and FBP rose to high levels at the lowest irradiance and then declined (Fig. 2). This response is especially marked in rice but is also clear for SBP in Arabidopsis. In both species the FBP/F6P and SBP/S7P ratios peaked at the lowest irradiance and then declined (Fig. 3). The peak of the SBP/S7P ratio at low irradiance was significant in both species in ANOVA performed on the combined data sets and on the data sets for individual species. The peak of the FBP/F6P ratio was significant in rice in ANOVA performed on the combined data sets and the rice data set, and in Arabidopsis in ANOVA performed on the Arabidopsis data set (Fig. 3; Supplementary Fig. S5). This response points to a selective restriction of flux at FBPase and SBPase in both species at irradiance around the respective light compensation point.

There were also marked differences between the two species. For example, the absolute levels of 3PGA and SBP were consistently and significantly higher in rice, whilst FBP and F6P were consistently and significantly higher in Arabidopsis (Fig. 2). Inspection of metabolite ratios (Fig. 3) reveals that the 3PGA/DHAP ratio was consistently higher and the DHAP/ RuBP ratio lower in rice than in Arabidopsis, indicating that the balance between the light reactions and RuBP regeneration is shifted to favour RuBP regeneration in rice. Except at the lowest irradiance, the FBP/F6P ratio was consistently lower and the SBP/S7P ratio consistently higher in rice than in Arabidopsis, with the interspecies differences often being significant (Fig. 3; see also Supplementary Fig. S5). This points to a shift in the balance between FBPase and SBPase activity, to favour the former in rice and probably the latter in Arabidopsis. Further, whereas in Arabidopsis, R5P, Ru5P+Xu5P, and RuBP were progressively and significantly higher, in rice they reached high values at 150 µmol m⁻² s⁻¹ and then did not increase much further (Fig. 2; Supplementary Fig. S2). This response indicates that rising rates of photosynthesis are driven by higher RuBP levels in Arabidopsis and by factors that stimulate RuBP utilization in rice.

Correlations between CBC intermediates

Correlation analyses were performed to identify sets of metabolites that show a similar response to rising irradiance, in either Arabidopsis alone (Fig. 4A, B), rice alone (Fig. 4C, D), or the combined Arabidopsis and rice data set (Fig. 4E, F). The analyses were performed with chlorophyll-normalized (Fig. 4A, C, E) and dimensionless (Fig. 4B, D, F) data sets. The analyses in Fig. 4 were performed with data sets that included the dark treatment; parallel analyses using data sets from which the dark treatment was excluded are provided in Supplementary Fig. S6. In analyses with chlorophyll-normalized data, most CBC intermediates correlated with each other in Arabidopsis, in rice, and in the combined data sets. The only exceptions are...
Fig. 1. Principal component (PC) analysis of the response of Arabidopsis and rice to rising irradiance. The plots show PC1 and PC2 for analyses performed on (A) chlorophyll-normalized data sets and (B) dimensionless data sets in which the amount of C in a given metabolite is normed on the total C in CBC intermediates plus 2PG in that sample (see legend to Supplementary Dataset S1 and Arrivault et al., 2019). Samples were collected in darkness and at 20, 80, 120, 280, and 440 µmol m$^{-2}$ s$^{-1}$ irradiance for Arabidopsis (shown in blue) and in darkness and at 50, 150, 250, 350, 450, 550, 650, and 730 µmol m$^{-2}$ s$^{-1}$ for rice (shown in red). Positions of individual samples at each irradiance are indicated by the corresponding number in small font. The average of these samples in the space defined by PC1 and PC2 is indicated by a circle (irradiance in large font) and the 95% confidence limits are depicted by bars. Metabolite loadings are depicted as grey arrows and the respective metabolite is shown in black font. The unusual asymmetric distribution of loadings in PC1 in the PC analysis with chlorophyll-normalized data indicates that a general increase in the levels of most metabolites contributes to the separation in PC1. PC analyses were also performed separately with the Arabidopsis and rice data sets (see Supplementary Figs S3 and S4, respectively).
FBP (especially in rice) and SBP (especially in Arabidopsis). Correlations were often weaker for rice, probably reflecting the plateauing of R5P, Ru5P+Xu5P, and RuBP at relatively low irradiance. Many correlations were weakened or disappeared in the dimensionless data sets, as expected because this normalization removes correlations that are driven by the general

Fig. 2. Response of selected CBC intermediates to rising irradiance in Arabidopsis and rice. (A) 3PGA (line), DHAP (dashed), (B) FBP (line), SBP (dashed), (C) F6P (line), S7P (dashed), (D) Xu5P+Ru5P (line), RuBP (dashed). The response of Arabidopsis is shown in the left-hand side (blue symbols) and of rice in the right-hand (red symbols) of each panel. In a given panel, the same y-axis scale is used for the two metabolites shown in the panel and for both species. Metabolites are normalized on total chlorophyll content. The results are shown as mean ±SD (n=4 in almost all cases; see Supplementary Dataset S1). One-way ANOVA with false discovery rate (FDR) was performed for each metabolite on the entire data set after log transformation, treating each species–irradiance combination as a separate treatment. This tests whether responses of a given species to changes in irradiance are significant, and whether metabolite levels differ between species whereby the latter comparison can be made independently of absolute irradiance. This was followed by a Tukey’s HSD post-hoc test. Treatments that are not significantly different share a letter. As two different metabolites are shown in each panel, the Tukey’s HSD results are shown with different cases for the two metabolites (e.g. ‘a’ and ‘A’). Letters are assigned such that ‘a’ or ‘A’ denotes the treatment group with the highest level. The original data are provided in Supplementary Dataset S1. Plots of all metabolites plus R5P and 2PG with scales selected to optimally view the response in a given species are provided in Supplementary Fig. S2A.
Fig. 3. Metabolite ratios. (A) 3PGA/DHAP, (B) DHAP/RuBP, (C) FBP/F6P, (D) SBP/S7P, (E) Ru5P+Xu5P/RuBP, and (F) RuBP/3PGA. The response of Arabidopsis is shown in the right-hand side (blue symbols) and of rice in the left-hand (red symbols) of each panel. In a given panel, the same y-axis scale is used for both species. The results are shown as mean ±SD (n=4 in almost all cases; see Supplementary Dataset S1). One-way ANOVA with FDR was performed on log-transformed data as in Fig. 2. This was followed by a Tukey’s HSD post-hoc test. Treatments that are not significantly different share a letter. Plots of these and further ratios with scales selected to optimally view the response in a given species are provided in Supplementary Fig. S5.
Fig. 4. Correlations between CBC intermediates and 2PG. (A, B) Arabidopsis, (C, D) rice, and (E, F) Arabidopsis and rice combined, using (A, C, E) chlorophyll-normalized data sets or (B, D, F) dimensionless data sets (see legend of Fig. 1) to exclude correlations that are driven by a general increase in metabolite levels. Spearman’s $r$ coefficient is denoted in the display and is visualized by a heatmap (red denotes a positive correlation and blue a negative correlation; see panel for scale which is the same for all panels). Non-significant correlations ($P > 0.05$) are not coloured. The original data are provided in Supplementary Dataset S1, and plotted in Fig. 2 and Supplementary Fig. S2A. This analysis used the samples from the dark and all irradiances. A similar analysis excluding dark samples is provided in Supplementary Fig. S6.
increase of metabolite levels as irradiance increases. Positive correlations were retained in Arabidopsis for DHAP versus F6P, FBP versus F6P, SBP versus FBP, SBP versus F6P, S7P versus R5P, and DHAP versus Ru5P+Xu5P, in rice for DHAP versus S7P, R5P versus Ru5P, Ru5P versus RuBP, 2PG versus DHAP, and 2PG versus S7P, and in the combined data sets for DHAP versus FBP, DHAP versus F6P; DHAP versus S7P, FBP versus F6P; FBP versus SBP, F6P versus S7P, R5P versus Ru5P+Xu5P, Ru5P+Xu5P versus RuBP, 2PG versus DHAP, and 2PG versus S7P. Several negative correlations appeared especially between 3PGA and other CBC intermediates.

Overall, these analyses reveal that although the absolute levels of metabolites vary between Arabidopsis and rice, the qualitative relationships between many CBC intermediates are rather conserved between these two species. The differing absolute levels may reflect differences in the balance between different reactions, whilst correlations common to both species may reflect conserved pathway structure.

Metabolites in pathways for end-product synthesis

The response of metabolites in end-product synthesis pathways is shown in Fig. 5 and Supplementary Fig. S2B. PC analyses with the entire metabolite data set recapitulated many features of the analyses with CBC intermediates (Supplementary Fig. S7). In particular, the lowest irradiance was an outlier in PC analyses with Arabidopsis (Supplementary Fig. S7A, B) and rice (Supplementary Fig. S7C, D), and Arabidopsis and rice showed different trajectories in PC analyses with the combined data sets (Supplementary Fig. S7E, F).

Inspection of the responses of individual metabolites to rising irradiance revealed some features that are shared in Arabidopsis and rice. In particular, there was a progressive and significant increase in dedicated intermediates for starch synthesis (ADPG; Fig. 5A) and sucrose synthesis (Suc6P; Fig. 5C) except for the highest irradiance in Arabidopsis. However, there were also species-dependent differences. G6P, UDPG (Fig. 5B), and G1P (Supplementary Fig. S2) were significantly lower and Suc6P (Fig. 5C) was significantly higher in rice than in Arabidopsis. Whereas PEP peaked at low irradiance and then declined and pyruvate rose progressively with irradiance in rice, PEP rose and pyruvate declined at high irradiance in Arabidopsis (Fig. 5D). These results point to cytosolic fluxes being regulated differently in Arabidopsis and rice.

Correlation analyses (Supplementary Fig. S8) revealed many correlations between metabolites of end-product synthesis, and between these metabolites and CBC intermediates in Arabidopsis. As in the analysis with CBC intermediates, many of the correlations were weakened or lost in the dimensionless data set, indicating that they are driven by a general increase in metabolite levels at higher irradiance. Correlations were weaker in rice, recapitulating the picture for CBC intermediates alone (see above, Fig. 4).

Response of metabolites in rice to low CO2

CBC intermediates remain high in Arabidopsis in low CO2 (Arrivault et al., 2009). To test if this is a general response, CBC and other intermediates were compared in rice leaves after 20 min at 500 ppm or 200 ppm CO2 (Fig. 6; original data provided in Supplementary Dataset S2). Decreasing the CO2 concentration to 200 ppm led to a small increase in 3PGA (significant; $P<0.05$), no significant change in RuBP (Fig. 6A), and small (<30%) non-significant decreases of metabolites involved in RuBP regeneration (Fig. 6A). There was a 2-fold ($P<0.01$) increase in 2PG (Fig. 6B), consistent with an increase in the rate of photorespiration. There were significant decreases in the levels of metabolites involved in end-product synthesis, including a 3-fold decrease ($P<0.01$) in ADPG (Fig. 6C), a smaller non-significant decreases in G6P, G1P, and Suc6P (Fig. 6D), and a significant ($P<0.01$) decrease of PEP (Fig. 6E).

Discussion

Differences in CBC operation between Arabidopsis and rice

Arrivault et al. (2019) reported that profiles of CBC intermediates vary between five C3 species, and proposed that there is interspecies variance in how the CBC operates. One potential issue with such studies is that as each species shows a different response of photosynthesis to irradiance, cross-species differences in metabolite profiles might be due to the conditions in which plants were harvested. Based on analyses at two irradiances in the C3 species maize and three irradiances in the C4 species Arabidopsis, Arrivault et al. (2019) concluded that irradiance did not have a strong impact on CBC intermediate profiles.

The current study compares CBC intermediate profiles over a larger irradiance range in two C3 species, the model eudicot Arabidopsis and the model monocot rice. Due to the differing light response of photosynthesis in Arabidopsis and rice (Supplementary Fig. S1), samples from a given irradiance cannot be directly compared but are better related to the light saturation response of the species. Material was harvested in the dark, close to the light compensation point, and at several irradiances up to ~90% and ~75% of that required to saturate photosynthesis in Arabidopsis and rice, respectively. PC analysis revealed that metabolite levels in Arabidopsis and rice were rather similar in darkness and very low light, but diverged as irradiance was increased (Fig. 1). This differing trajectory shows that Arabidopsis and rice use partly differing strategies to increase CBC flux.

Loading of metabolites in the PC analysis (Fig. 1) and inspection of the responses of individual metabolites (Figs 2, 3) revealed that several factors drive the separation of Arabidopsis and rice. First, there was a lower 3PGA/triose-P ratio and higher triose-P/RuBP ratio in Arabidopsis than in rice, indicating that the balance between the light reactions and RuBP regeneration is shifted in favour of the light reactions in Arabidopsis and RuBP regeneration in rice. Secondly, except at very low irradiance, there was a consistent trend to a higher FBP/F6P ratio and lower SBP/S7P ratio in Arabidopsis compared with rice, pointing to a shift in the balance between FBPase and SBPase to favour FBPase in rice and SBPase in Arabidopsis. Thirdly, CBC intermediates were more strongly correlated with each other in Arabidopsis than in rice (Fig. 4),
indicating that increasing CBC flux is driven by a general increase in metabolite levels in Arabidopsis, whereas regulation of individual enzymes plays a larger role in rice.

One specific example where metabolites were more strongly correlated in Arabidopsis than rice concerns the later steps in RuBP regeneration. Whereas in Arabidopsis the levels of R5P, Ru5P+Xu5P, and RuBP rose progressively with irradiance, in rice R5P, Ru5P+Xu5P, and RuBP rose to high levels at relatively low irradiance and then plateaued. An earlier study with rice also reported that RuBP levels plateaued at lower irradiances.
than those needed to saturate photosynthesis (Makino et al., 1985). Rising irradiance leads to higher rates of photosynthesis and, hence, of RuBP regeneration and utilization. Our results indicate that this increase in RuBP utilization is largely driven by increased RuBP in Arabidopsis, whilst further factors contribute in rice. Earlier studies also pointed to interspecies variation in the response of RuBP to rising irradiance; for example, RuBP levels rose progressively in bean leaves (Badger et al., 1984) and Chlamydomonas (Mettler et al., 2014), whereas RuBP plateaued at irradiances at which photosynthesis was still increasing in wheat (Perchorowicz et al., 1981), spinach (Dietz et al., 1984), and radish (von Caemmerer and Edmondson, 1986) leaves. The latter response resembles that in rice, and points to flux in these species being increased by removing Rubisco inhibitors and/or increasing Rubisco activation.

Chlorophyll and protein content per unit leaf mass are higher in rice than in Arabidopsis. Rice has an unusual leaf anatomy with small lobed mesophyll cells, a high density of chloroplasts with proliferous stromules at the cell surface, and mitochondria located in the inner part of the cell (Sage and Sage, 2009). This increases mesophyll conductance and promotes recapture of photorespired CO2 (Sage and Sage, 2009; Busch et al., 2013). Rice also differs from Arabidopsis in having lobe-shaped guard cells and subsidiary cells. These are typical for grasses and allow more efficient CO2 exchange with the atmosphere (Hetherington and Woodward, 2003; Franks and Farquhar, 2007; Raissig et al., 2016, 2017). These anatomical and ultrastructural features may explain how rice can function efficiently with a higher leaf chlorophyll and protein content, which would otherwise tend to draw down internal CO2, increase RuBP oxygenation relative to carboxylation, and decrease photosynthetic efficiency. 2PG levels and the RuBP/2PG ratio in rice resembled those in Arabidopsis over much of the light saturation response (Supplementary Figs S3, S7).
S5), which is consistent with photorespiration not being higher in rice. Further studies are required to learn to what extent anatomical and ultrastructural adaptations facilitate changes in CBC operation in rice.

There were also differences between Arabidopsis and rice in the responses of metabolites in end-product synthesis (Fig. 5; Supplementary Figs S7, S8). In Arabidopsis, most metabolites in end-product synthesis rose progressively, correlating with CBC intermediates. In rice the response was more diverse; for example, G6P and G1P did not increase with rising irradiance. Further, G6P and UDPG levels were lower and Suc6P levels were higher in rice than in Arabidopsis. These observations point to differences in how sucrose synthesis is regulated, for example a larger role for sucrose-phosphate synthase in rice. There are also implications for CBC operation. The G6P pool typically contains a large part of the total P in photosynthetic metabolites (see Fig. 2 and Szecowka et al. (2013) for Arabidopsis, Dietz and Heber (1984), Stitt et al. (1984a, b), and Gerhardt et al. (1987) for spinach, Stitt et al. (1983) for wheat, and Badger et al. (1984) for bean]. The small G6P pool in rice may allow larger cytosolic pools of triose-P and 3PGA, which may affect triose-P export from the chloroplast and energy shuttling via the triose-P/3PGA shuttle.

Shared features in the response to rising irradiance

There are also shared features in the response of Arabidopsis and rice to increasing irradiance. First, both species show a general increase in the levels of many metabolites as irradiance increases, including 3PGA, triose-P, F6P, and S7P over most of the irradiance range, and R5P, Ru5P+Xu5P, and RuBP in the lower part of the irradiance response (Fig. 2; Supplementary Fig. S2B). Secondly, from the light compensation point on, increasing irradiance led to a slight decline in the 3PGA/triose-P ratio and a slight rise in the triose-P/RuBP ratio. However, in both species, this shift was small compared with the massive rise in CBC flux. Whilst rising irradiance drives 3PGA reduction by supplying more ATP and NADPH, regulation of the CBC enzymes will be needed to speed up conversion of triose-P to RuBP.

Thirdly, in both species, triose-P rose sharply between darkness and the light compensation point, where triose-P already reached a level that was about one-fifth (Arabidopsis) or one-third (rice) of that in high irradiance (Fig. 2). The CBC is an autocatalytic pathway and if CBC intermediates fell to very low levels in low light, time would be needed to build metabolite pools up again after moving back to high light. Maintenance of relatively high levels of CBC intermediates in low light will allow a rapid increase in CBC flux and photosynthesis after sudden increases in light intensity due to changing cloud cover and, especially, canopy movement (Gibbs et al., 2018; Burgess et al., 2019). A rapid response of photosynthetic rate to a rise in light intensity is thought to be an important factor for photosynthetic efficiency in the dense canopies used in modern agriculture (Pearcy et al., 1990; Taylor and Long, 2017; Burgess et al., 2019). The regulatory network that maintains these relatively high levels of metabolites in low light will be discussed in the next subsection.

A fourth shared feature is that FBP and especially SBP rose to a high level or even peaked in very low irradiance (Fig. 2), and that the FBP/F6P and SBP/S7P ratios peaked in low irradiance and then declined (Fig. 3). These results point to FBPase and SBPase restricting CBC flux at irradiances around the light compensation point, presumably due to incomplete redox activation of these enzymes. As mentioned in the Introduction, wasteful futile cycles will occur if enzymes such as FBPase and SBPase are active in the dark when respiratory pathways are operating. Futile cycles would also be especially deleterious in low irradiance, when they would substantially decrease the efficiency with which light energy is used to drive CO2 fixation, resulting in a lower quantum yield and higher light compensation point.

After relaxing from the low irradiance peak, the FBP/F6P and SBP/S7P ratios remained rather constant (Arabidopsis) or continued to fall (rice) across a wide range of irradiance, even though the rate of photosynthesis rose many fold. This implies that CBC fluxes may be promoted by rising redox activation of FBPase and SBPase over a quite broad irradiance range, partly explaining how CBC flux is speeded up as the light intensity rises.

Redox activation of FBPase and SBPase increases strongly between darkness and high light (Buchanan and Balmer, 2005; Heldt et al., 2005). There is, however, little information on the irradiance range over which this occurs. This is partly because it is technically challenging to extract and assay these enzymes without altering their redox state (Laing et al., 1981; Wirtz et al., 1982). New MS-based methods to quantify redox states of cysteine in proteins (Lennicke et al., 2016; Zhang et al., 2016) will allow testing of these predictions, and may contribute important new insights into CBC regulation.

Our findings are supported by earlier, albeit more fragmentary, studies of CBC intermediate levels in other species (see Supplementary Table S1). For example, studies in wheat proto-plasts (Stitt et al., 1983) and leaves of bean (Badger et al., 1984) and spinach (Dietz et al., 1984; Stitt et al., 1984a) reported a general increase in metabolite levels with rising irradiance, relatively small changes in the 3PGA/triose-P and triose-P/RuBP ratios with rising irradiance, a relatively high level of triose-P at low irradiance, and a high level or peak of FBP at low irradiance. A more recent study in C. reinhardtii reported marked peaks of FBP and SBP and high FBP/F6P and SBP/S7P ratios in low irradiance (Mettler et al., 2014).

Shared features in the response to low CO2

Comparison of Fig. 6 with published metabolite profiles for Arabidopsis (Arrivault et al., 2009) reveals that rice and Arabidopsis respond in a similar manner to low CO2, with only a slight decrease in RuBP and metabolites involved in its regeneration. A similar picture emerges from earlier more fragmentary analyses of the response to low CO2 in wheat proto-plasts (Stitt et al., 1983) and bean and spinach leaves (Badger et al., 1984; Dietz and Heber, 1984), and to treatments such as salinity, water stress, or abscisic acid application that lead to stomatal closure and low intracellular [CO2] (c) (Seemann and Sharkey, 1986, 1987; Sharkey and Seemann, 1989).
The relatively high levels of CBC intermediates in low CO₂ and under stress conditions that lead to low internal CO₂ imply that end-product synthesis has been inhibited, and this idea is supported by the decline of dedicated metabolites for sucrose and starch synthesis. Maintenance of high levels of CBC intermediates allows continued flux in the CBC to generate RuBP that is oxygenated to 2PG and metabolized via photorespiration. This provides a way in which to dissipate energy in these stressful conditions (see Arrivault et al., 2009; Foyer et al., 2009; Voss et al., 2013).

As outlined in the Introduction, a regulatory network around the cytosolic FBPase involving fructose 2,6-bisphosphate restricts removal of triose-P from the CBC until a threshold concentration of triose-P is reached, and facilitates a large increase in flux to sucrose when this threshold is exceeded (Herzog et al., 1984; Stitt et al., 1987, 2010; Stitt, 1990). The conserved response of CBC intermediate levels to low CO₂ indicates that this represents a general principle across many species. The observation that triose-P and other CBC intermediates are maintained at relatively high levels at the light compensation point in many species (see the previous section) also points to widespread operation of this regulatory mechanism. In addition to sucrose synthesis, flux to starch needs to be restricted, especially in species where starch is a major product of photosynthesis. Allostereic regulation of ADP glucose pyrophosphorylase by the 3PGA/Pi ratio (Ballicora et al., 2004) will restrict starch synthesis in low irradiance, when most phosphorylated CBC intermediates decline and stromal Pi is likely to be high. Allosteric regulation may be less effective in low CO₂, when CBC intermediates show a smaller decrease than in low light. In these conditions, plastid glucan phosphorylase may contribute by remobilizing starch; this will decrease the net rate of starch synthesis and recycle carbon to maintain CBC metabolite levels (Weise et al., 2006). Whilst starch degradation is under circadian regulation (Stitt and Zeeman, 2012), water stress induces a novel starch degradation pathway involving α-AMYLASE3 (Zanella et al., 2016). This may bypass regulation by the circadian clock and provide glucans that can be degraded by plastid phosphorylase.

Interestingly, recent pioneering studies in which the CBC was introduced into Escherichia coli showed that selective evolution was required to allow CO₂-dependent growth of E. coli, and that several of the underlying mutations involved a decrease in the activity of enzymes that withdraw intermediates from the CBC (Antonovsky et al., 2017; Herz et al., 2017). This finding underlines that CBC operation depends on regulation of the rate at which intermediates exit this autocatalytic cycle.

In conclusion, the CBC exhibits both contrasting and shared features between the model eudicot Arabidopsis and the model monocot rice. Contrasting features include a shift between the light reactions and RuBP regeneration, a shift in the balance between FBPase and SBPase, and differences in the regulation of RuBP utilization, which depends strongly on RuBP levels in Arabidopsis and factors that promote Rubisco activity in rice. These differences may be due to interspecies variation in protein abundance, post-translational regulation, or the regulatory properties of enzymes. From the viewpoint of crop improvement, these findings imply that the best targets to increase CBC flux may differ between species. However, our results also reveal shared features between Arabidopsis and rice, and inspection of the fragmentary information for other species indicates that these features may be more widely conserved. One is a restriction on flux at FBPase and SBPase at low irradiance, indicating incomplete post-translational activation of these enzymes. Another is maintenance of high levels of CBC intermediates at the light or CO₂ compensation point, providing evidence for effective inhibition of end-product synthesis when net C fixation is low. These shared features may be important for efficient CBC function in suboptimal or fluctuating conditions. For example, restricting FBPase and SBPase activity in low irradiance may suppress wasteful futile cycles and improve photosynthetic efficiency in low light. Maintenance of metabolites at relatively high levels in low light or low CO₂ will keep the CBC ‘poised to go’, allowing a rapid response when irradiance suddenly increases and supporting rapid RuBP regeneration, photorespiration, and energy dissipation when stomata are closed.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Light saturation response of photosynthesis in Arabidopsis and rice.

Fig. S2. Metabolite levels displayed on scales to optimally display the irradiance response for each species, and with ANOVA performed on the Arabidopsis and rice data sets separately.

Fig. S3. Principal component (PC) analysis of CBC intermediates and 2PG on the Arabidopsis data set.

Fig. S4. Principal component (PC) analysis of CBC intermediates and 2PG on the rice data set.

Fig. S5. Metabolite ratios displayed on scales to optimally display the irradiance response for each species, and with one-way ANOVA performed on the Arabidopsis and rice data sets separately.

Fig. S6. Correlation analysis of CBC intermediates and 2PG on the Arabidopsis and rice data sets (excluding dark samples).

Fig. S7. Principal component (PC) analysis of all metabolites on Arabidopsis and rice data sets.

Fig. S8. Correlation analysis for all metabolites in the Arabidopsis and rice data sets.

Table S1. Analysis of published responses of metabolites to changing irradiance or CO₂ concentration.

Dataset S1. Metabolite levels and metabolite ratios in Arabidopsis and rice at different irradiances, and statistical analyses of the combined Arabidopsis and rice data sets by one-way ANOVA.

Dataset S2. Metabolite levels and metabolite ratios in rice at different CO₂ concentrations.

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References

Antonovsky N, Gleizer S, Milo R. 2017. Engineering carbon fixation in E. coli: from heterologous Rubisco expression to the Calvin–Benson–Bassham cycle. Current Opinion in Biotechnology 47, 83–91.

Arnold A, Nikoloski Z. 2011. A quantitative comparison of Calvin–Benson cycle models. Trends in Plant Science 16, 676–683.

Arnold A, Nikoloski Z. 2012. In search for an accurate model of the photosynthetic carbon metabolism. Mathematics and Computers in Simulation 96, 171–194.

Arrivault S, Alexandre Moraes T, Obata T, et al. 2019. Metabolite profiles reveal interspecific variation in operation of the Calvin–Benson cycle in both C3 and C4 plants. Journal of Experimental Botany 70, 1843–1858.

Arrivault S, Guenther M, Fry SC, Fuenfgeld MM, Veyel D, Mettler-Altman T, Stitt M, Lunn JE. 2015. Synthesis and use of stable-isotope-labeled internal standards for quantification of phosphorylated metabolites by LC-MS/MS. Analytical Chemistry 87, 6896–6904.

Arrivault S, Guenther M, Ivakov A, Feil R, Voslosh d, van Dongen JT, Sulpic R, Stitt M. 2009. Use of reverse-phase liquid chromatography, linked to tandem mass spectrometry, to profile the Calvin cycle and other metabolic intermediates in Arabidopsis rosellata at different carbon dioxide concentrations. The Plant Journal 59, 826–839.

Ashton AR, Hatch MD. 1983. Regulation of C4 photosynthesis: physical and kinetic properties of active (dithiol) and inactive (disulfide) NADP-malate dehydrogenase from Zsma mays. Archives of Biochemistry and Biophysics 227, 406–415.

Ashton AR, Trevanion SJ, Carr PD, Verger D, Ollis DL. 2000. Structural and kinetic properties of active (dithiol) and inactive (disulfide) NADP-malate dehydrogenase from Limonium. Archives of Biochemistry and Biophysics 372, 53–64.

Bassham JA, Krause GH. 1969. Free energy changes and metabolic reduction-cycle intermediates. Planta 100, 305–313.

Baker JT, Allen LH, Boote KJ, Jones P, Jones JW. 1990. Rice photosynthesis and evapotranspiration in subambient, ambient, and superambient carbon dioxide concentrations. Agronomy Journal 82, 834–840.

Ballicora MA, Iglesias AA, Preiss J. 2004. ADP-glucose pyrophosphorylase: a regulatory enzyme for plant starch synthesis. Photosynthesis Research 79, 1–24.

Bassham JA, Krause GH. 1969. Free energy changes and metabolic regulation in steady-state photosynthetic carbon reduction. Biochimica et Biophysica Acta 189, 207–221.

Betti M, Bauwe H, Busch FA, et al. 2016. Manipulating photosynthesis to increase plant productivity: recent advances and perspectives for crop improvement. Journal of Experimental Botany 67, 2977–2988.

Buchanan BB, Balmer Y. 2005. Redox regulation: a broadening horizon. Annual Review of Plant Biology 56, 187–220.

Burgess AJ, Gibbs JA, Murchie EH. 2019. A canopy comundrum: can wind-induced movement help to increase crop productivity by relieving photosynthetic limitations? Journal of Experimental Botany 70, 2371–2380.

Busch FA, Sage TL, Cousins AB, Sage RF. 2013. C3 plants enhance rates of photosynthesis by reassimilating photorespired and respirated CO2. Plant, Cell & Environment 36, 200–212.

Cruz JA, Emery C, Wüst M, Kramer DM, Lange BM. 2008. Metabolite profiling of Calvin cycle intermediates by HPLC-MS using mixed-mode stationary phases. The Plant Journal 55, 1047–1060.

Dietz KJ, Heber U. 1984. Rate-limiting factors in leaf photosynthesis. I. Carbon fluxes in the Calvin cycle. Biochimica et Biophysica Acta 767, 432–443.

Dingkuhn M, Schnier HF, de Datta SK, Wijangco E, Dörffling K. 1990. Measurement of subcellular metabolite levels in leaves by fractionation of freeze-stopped material in nonaqueous media. Plant Physiology 95, 542–547.

Dingkuhn M, Schnier HF, de Datta SK, Wijangco E, Dörffling K. 1990. Measurement of subcellular metabolite levels in leaves by fractionation of freeze-stopped material in nonaqueous media. Plant Physiology 95, 542–547.

Giber JA, Murchie EH. 2019. A canopy comundrum: can wind-induced movement help to increase crop productivity by relieving photosynthetic limitations? Journal of Experimental Botany 70, 2371–2380.

Gibson Y, Vigeolas H, Tiessen A, Geigenberger P, Stitt M. 200. Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADPGLc, nucleotide phosphates, and glycolytic intermediates based on a novel enzymic cycling system. The Plant Journal 30, 221–235.

Gontier MA, Mabberly SC. 2012. An intrinsically disordered protein, CP12: jack of all trades and master of the Calvin cycle. Biochemical Society Transactions 40, 995–999.

Haake V, Geiger M, Balmer Y, Engels C, Zrenner R, Stitt M. 1999. Changes in aldolase activity in wild-type potato plants are important for acclimation to growth irradiance and carbon dioxide concentration, because plastid aldolase exerts control over the ambient rate of photosynthesis across a range of growth condition. The Plant Journal 17, 479–489.

Haake V, Zrenner R, Sonnewald U, Stitt M. 1998. A moderate decrease of plastid aldolase activity inhibits photosynthesis, alters the levels of sugars and starch, and inhibits growth of potato plants. The Plant Journal 14, 147–157.

Hagemann M, Kern R, Maurino VG, Hanson DT, Weber AP, Sage RF, Bauwe H. 2016. Evolution of photosorption from cyanobacteria to land plants, considering protein phylogenies and acquisition of carbon concentrating mechanisms. Journal of Experimental Botany 67, 2963–2976.

Hasunuma T, Harada K, Miyazawa S, Kondo A, Fukusaki E, Miyake C. 2005. Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADPGLc, nucleotide phosphates, and glycolytic intermediates based on a novel enzymic cycling system. The Plant Journal 30, 221–235.

Heldt HW, Piechulla B, Heldt F. 2005. Plant biochemistry. Cambridge, MA: Academic Press.
Herz E, Antonovsky N, Bar-On Y, et al. 2017. The genetic basis for the adaptation of E. coli to sugar synthesis from CO₂. Nature Communications 8, 1705.

Herzog B, Stitt M, Heldt HW. 1984. Control of photosynthetic sucrose synthesis by fructose 2,6-bisphosphate: II. Properties of the cytosolic fructose 1,6-bisphosphatase. Plant Physiology 75, 561–565.

Hetherington AM, Woodward FL. 2003. The role of stomata in sensing and driving environmental change. Nature 424, 6951–6951.

Jelitto T, Sonnewald U, Willmitzer L, Hajirezaei M, Stitt M. 1992. Inorganic pyrophosphate content and metabolites in potato and tobacco plants expressing E. coli pyrophosphatase in their cytosol. Planta 188, 238–244.

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.

Laing WA, Stitt M, Heldt HW. 1981. Changes in the activity of ribulosebisphosphate kinase and fructose- and sedoheptulose-bisphosphatase in chloroplasts. Biochimica et Biophysica Acta 637, 349–359.

Laisk A, Eichelmann H, Oja V. 2006. C₃ photosynthesis in silico. Photosynthesis Research 90, 45–66.

Lawson T, Kramer DM, Raines CA. 2012. Improving yield by exploiting mechanisms underlying natural variation of photosynthesis. Current Opinion in Biotechnology 23, 215–220.

Lefebvre S, Lawson T, Zakheniuk OV, Lloyd JC, Raines CA, Fryer M. 2005. Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. Plant Physiology 138, 451–460.

Lennicke C, Rahn J, Heimer N, Lichtenfels R, Wessjohann LA, Selliger B. 2016. Redox proteomics: methods for the identification and enrichment of redox-modified proteins and their applications. Proteomics 16, 197–213.

Long SP, Marshall-Colon A, Zhu XG. 2015. Meeting the global food demand of the future by engineering crop photosynthesis and yield potential. Cell 161, 56–66.

Long SP, Zhu XG, Naidu SL, Ort DR. 2006. Can improvement in photosynthesis increase crop yields? Plant, Cell & Environment 29, 315–330.

López-Calcagno PE, Howard TP, Raines CA. 2014. The CP12 protein family: a threonine-mediated metabolic switch? Frontiers in Plant Science 5, 9.

Lorimer GH. 1981. The carboxylation and oxygenation of ribulose-1,5-bisphosphate: the primary events in photosynthesis and photorespiration. Annual Review of Plant Physiology 32, 349–382.

Lunn JE, Feil R, Hendriks JH, Gibon Y, Morcuende R, Osuna D, Scheible WR, Carillo P, Hajirezaei MR, Stitt M. 2006. Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in potato. Planta 166, 414–420.

McClain AM, Sharkey TD. 2019. Triose phosphate utilization and beyond: from photosynthesis to end product synthesis. Journal of Experimental Botany 70, 1755–1766.

Merlo L, Geigenberger P, Hajirezaei M, Stitt M. 1993. Changes of carbohydrates, metabolites and enzyme activities in potato tubers during development, and within a single tuber along astolom–apex gradient. Journal of Plant Physiology 142, 392–402.

Mettler T, Mühlhaus T, Hemme D, et al. 2014. Systems analysis of the response of photosynthesis, metabolism, and growth to an increase in infraredance in the photosynthetic model organism Chlamydomonas reinhardtii. The Plant Cell 26, 2310–2350.

Moore BD, Isidoro E, Seemann JR. 1993. Distribution of 2-carboxyarabinitol among plants. Phytochemistry 34, 703–707.

Murchie EH, Chen YZ, Hubbart S, Peng S, Horton P. 1999. Interactions between senescence and leaf orientation determine in situ patterns of photosynthesis and photoinhibition in field-grown rice. Plant Physiology 119, 553–564.

Ort DR, Merchant SS, Alric J, et al. 2015. Redesigning photosynthesis to sustainably meet global food and bioenergy demand. Proceedings of the National Academy of Sciences, USA 112, 8530–8536.

Osmond CB. 1981. Photorespiration and photoinhibition: some implications for the energetics of photosynthesis. Biochimica et Biophysica Acta 639, 77–98.

Parry MA, Keys AJ, Madgwick PJ, Carmo-Silva AE, Andralojc PJ. 2008. Rubisco regulation: a role for inhibitors. Journal of Experimental Botany 59, 1569–1580.

Pearcy R. 1990. Sunflecks and photosynthesis in plant canopies. Annual Review of Plant Physiology and Plant Molecular Biology 41, 421–453.

Perchorowicz JT, Rayes NA, Jensen RG. 1981. Light limitation of photosynthesis and activation of ribulose bisphosphate carboxylase in wheat seedlings. Proceedings of the National Academy of Science, USA 78, 2985–2989.

Petersson G, Ryde-Pettersson U. 1989. A mathematical model of the Calvin photosynthesis cycle. European Journal of Biochemistry 175, 661–672.

Poolman MG, Fell DA, Thomas S. 2000. Modelling photosynthesis and its control. Journal of Experimental Botany 51, 319–328.

Portis AR Jr, Li C, Wang D, Salvucci ME. 2008. Regulation of Rubisco activase and its interaction with Rubisco. Journal of Experimental Botany 59, 1597–1604.

Portis AR Jr, Parry MA. 2007. Discoveries in Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase): a historical perspective. Photosynthesis Research 94, 121–143.

Prins A, Orr DJ, Andralojc PJ, Reynolds MP, Carmo-Silva E, Parry MA. 2016. Rubisco catalytic properties of wild and domesticated relatives provide scope for improving wheat photosynthesis. Journal of Experimental Botany 67, 1827–1838.

Raines CA, Harrison EP, Ölger H, Lloyd JC. 2000. Investigating the role of the thiol-regulated enzyme sedoheptulose-1,7-bisphosphatase in the control of photosynthesis. Physiologia Plantarum 110, 303–308.

Raisig MT, Abrash E, Bettadapura A, Vogel JP, Bergmann DC. 2016. Grasses use an alternatively wired bHLH transcription factor network to establish stomatal identity. Proceedings of the National Academy of Sciences, USA 113, 8326–8331.

Raisig MT, Matos JL, Ximena M, et al. 2017. Supplementary materials for mobile MUTE specifies subsidiary cells to build physiologically improved grass starcha. Science 355, 1215.

Raven JA, Beardall J, Sánchez-Baracaldo P. 2017. The possible evolution and future of C₄-concentrating mechanisms. Journal of Experimental Botany 68, 3701–3716.

Sage RF. 2017. A portrait of the C₄ photosynthetic family on the 50th anniversary of its discovery: species number, evolutionary lineages, and Hall of Fame. Journal of Experimental Botany 68, e11–e28.

Sage TL, Sage RF. 2009. The functional anatomy of rice leaves: implications for photosynthesis and CO₂ and C₄ photosynthesis into rice. Plant & Cell Physiology 50, 756–772.

Scheible R. 1991. Redox-modulation of chloroplast enzymes: a common principle for individual control. Plant Physiology 96, 1–3.

Schimkat D, Heineke D, Heldt HW. 1988. A mathematical model of photorespiration and photoinhibition in field-grown rice. Plant Physiology 89, 855–860.

Seemann JR, Sharkey TD. 1986. Salinity and nitrogen effects on photosynthesis, ribulose-1,5-bisphosphate carboxylase and metabolite pool sizes in Phaseolus vulgaris L. Plant Physiology 82, 555–560.

Seemann JR, Sharkey TD. 1987. The effect of abscisic acid and other inhibitors on photosynthetic capacity and the biochemistry of CO₂ assimilation. Plant Physiology 84, 606–700.

Seliger B. 2017. A portrait of the C₄ photosynthetic family on the 50th anniversary of its discovery: species number, evolutionary lineages, and Hall of Fame. Journal of Experimental Botany 68, e11–e28.

Sharkey TD. 1985a. Photosynthesis in intact leaves of C₄ plants. The Botanical Review 5, 53–105.
Sharkey TD. 1985b. O2-insensitive photosynthesis in C3 plants: its occurrence and a possible explanation. Plant Physiology 78, 71–75.

Sharkey TD. 1988. Estimating the rate of photorespiration in leaves. Physiologia Plantarum 73, 147–152.

Sharkey TD, Seemann JR. 1989. Mid water stress effects on carbon-reduction-cycle intermediates, ribulose bisphosphate carboxylase activity, and spatial homogeneity of photosynthesis in intact leaves. Plant Physiology 89, 1060–1065.

Sharwood RE, Sonawane BV, Ghannoum O, Whitney SM. 2016. Improved analysis of C4 and C3 photosynthesis via refined in vitro assays of their carbon fixation biochemistry. Journal of Experimental Botany 67, 3137–3148.

Simkin AJ, Lopez-Calcaigo PE, Davey PA, Headland LR, Lawson T, Timm S, Bauwe H, Raines CA. 2017. Simultaneous stimulation of sedoheptulose 1,7-bisphosphatase, fructose 1,6-bisphosphate aldolase and the photorespiratory glycine decarboxylase-H protein increases CO2 assimilation, vegetative biomass and seed yield in Arabidopsis. Plant Biotechnology Journal 15, 805–816.

South PF, Cavanagh AP, Liu HW, Ort DR. 2019. Synthetic glycolate metabolism pathways stimulate crop growth and productivity in the field. Science 363, eaat9077.

Stitt M. 1990. Fructose-2,6-bisphosphate as a regulatory molecule in plants. Annual Review of Plant Physiology and Plant Molecular Biology 41, 153–185.

Stitt M. 1999. The first will be last and the last will be first: non-regulated enzymes call the tune? In: Burrell JA, Bryant MM, Kruger NJ, eds. Plant carbohydrate biochemistry. Oxford: BIOS Scientific Publishers, 1–16.

Stitt M, Herzog B, Heldt HW. 1984a. Control of photosynthetic sucrose synthesis by fructose 2,6-bisphosphate. I. Coordination of CO2 fixation and sucrose synthesis. Plant Physiology 75, 548–553.

Stitt M, Huber S, Kerr P. 1987. Control of photosynthetic sucrose formation. In: Hatch MD, Boardman NK, eds. Photosynthesis, a comprehensive treatise. Academic Press, 327–409.

Stitt M, Kürzel B, Heldt HW. 1984b. Control of photosynthetic sucrose synthesis by fructose 2,6-bisphosphate. II. Partitioning between sucrose and starch. Plant Physiology 75, 554–560.

Stitt M, Lilley RM, Heldt HW. 1982a. Adenine nucleotide levels in the cytosol, chloroplasts, and mitochondria of wheat leaf protoplasts. Plant Physiology 70, 971–977.

Stitt M, Lunn J, Usadel B. 2010. Arabidopsis and primary photosynthetic metabolism—more than the icing on the cake. The Plant Journal 61, 1067–1091.

Stitt M, Mieskes G, Soling HD, Heldt HW. 1982b. On a possible role of fructose 2,6-bisphosphate in regulating photosynthetic metabolism in leaves. FEBS Letters 145, 217–222.

Stitt M, Schulze D. 1994. Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. Plant, Cell & Environment 17, 465–487.

Stitt M, Sonnewald U. 1995. Regulation of metabolism in transgenic plants. Annual Review of Plant Physiology and Plant Molecular Biology 46, 341–368.

Stitt M, Sulipce R, Gibson Y, Whitwell R, Skilbeck R, Parker S, Ellison R. 2007. Cryogenic grinder system. German Patent No. 08146.0025U1. MPG/SFX Link Resolver.

Stitt M, Wirtz W, Heldt HW. 1983. Regulation of sucrose synthesis by cytoplasmic fructosebisphosphatase and sucrose phosphate synthase during photosynthesis in varying light and carbon dioxide. Plant Physiology 72, 767–774.

Stitt M, Zeeman SC. 2012. Starch turnover: pathways, regulation and role in growth. Current Opinion in Plant Biology 15, 282–292.

Szecowka M, Heise R, Tohge T, et al. 2013. Metabolic fluxes in an illuminated Arabidopsis rosette. The Plant Cell 25, 694–714.

Taylor SH, Long SP. 2017. Slow induction of photosynthesis on shade to sun transitions in wheat may cost at least 21% of productivity. Philosophical Transactions of the Royal Society B: Biological Sciences 372, 20160543.

von Caemmerer S, Edmondson DL. 1986. Relationship between steady-state gas exchange, in vivo ribulose bisphosphate carboxylase activity and some carbon reduction cycle intermediates in Raphanus sativus. Functional Plant Biology 13, 669–688.

von Caemmerer S, Farquhar GD. 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153, 376–387.

Voss I, Sunil B, Scheibe R, Raghavendra AS. 2013. Emerging concept for the role of photorespiration as an important part of abiotic stress response. Plant Biology 15, 713–722.

Weise SE, Schrader SM, Kleinbeck KR, Sharkey TD. 2006. Carbon balance and circadian regulation of hydrolytic and phosphorolytic breakdown of transitory starch. Plant Physiology 141, 879–886.

Wirtz W, Stitt M, Heldt HW. 1982. Light activation of Calvin cycle enzymes as measured in pea leaves. FEBS Letters 142, 223–226.

Wullschlegler SD. 1993. Biochemical limitations to carbon assimilation in C3 plants—a retrospective analysis of the A/Ci curves from 109 species. Journal of Experimental Botany 44, 907–920.

Yeoh HH, Badger MR, Watson L. 1980. Variations in Km(CO2) of ribulose-1,5-bisphosphate carboxylase among grasses. Plant Physiology 66, 1110–1112.

Zanella M, Borghi GL, Pirone C, Thalmann M, Pazmino D, Costa A, Santelia D, Tost P, Scharf P. 2016. α-Amylase 1 (BAM1) degrades transitory starch to sustain proline biosynthesis during drought stress. Journal of Plant Physiology 191, 1–7.

Zhu XG, de Sturler E, Long SP. 2007. Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: a numerical simulation using an evolutionary algorithm. Plant Physiology 145, 513–526.