Metabolite profiles reveal interspecific variation in operation of the Calvin–Benson cycle in both C4 and C3 plants

Stéphanie Arrivault, Thiago Moraes, Toshihiro Obata, David Medeiros, Alisdair Fernie, Alix Boulouis, Martha Ludwig, John Lunn, Gian Luca Borghi, Armin Schlereth, et al.

To cite this version:

Stéphanie Arrivault, Thiago Moraes, Toshihiro Obata, David Medeiros, Alisdair Fernie, et al.. Metabolite profiles reveal interspecific variation in operation of the Calvin–Benson cycle in both C4 and C3 plants. Journal of Experimental Botany, Oxford University Press (OUP), 2019, 70 (6), pp.1843-1858. 10.1093/jxb/erz051 . hal-02122379
RESEARCH PAPER

Metabolite profiles reveal interspecific variation in operation of the Calvin–Benson cycle in both C₄ and C₃ plants

Stéphanie Arrivault, Thiago Alexandre Moraes, Toshihiro Obata, David B. Medeiros, Alisdair R. Fernie, Alix Boulouis, Martha Ludwig, John E. Lunn, Gian Luca Borghi, Armin Schlereth, Manuela Guenther, Mark Stitt, Stéphanie Arrivault, Armin Schlereth, and Mark Stitt

1 Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, D-14476 Potsdam-Golm, Germany
2 School of Molecular Sciences, The University of Western Australia, 35 Stirling Hwy, Crawley WA 6009, Australia
3 Department of Biochemistry, Center for Plant Science Innovation, University of Nebraska-Lincoln, 1901 Vine Str, Lincoln, NE 68588, USA.
4 Present address: Institut de Biologie Physico-Chimique, CNRS - Sorbonne Université, Paris, France.

Received 2 October 2018; Editorial decision 22 January 2019; Accepted 29 January 2019

Editor: Christine Raines, University of Essex, UK

Abstract

Low atmospheric CO₂ in recent geological time led to the evolution of carbon-concentrating mechanisms (CCMs) such as C₄ photosynthesis in >65 terrestrial plant lineages. We know little about the impact of low CO₂ on the Calvin–Benson cycle (CBC) in C₃ species that did not evolve CCMs, representing >90% of terrestrial plant species. Metabolite profiling provides a top-down strategy to investigate the operational balance in a pathway. We profiled CBC intermediates in a panel of C₄ (Zea mays, Setaria viridis, Flaveria bidentis, and F. trinervia) and C₃ species (Oryza sativa, Triticum aestivum, Arabidopsis thaliana, Nicotiana tabacum, and Manihot esculenta). Principal component analysis revealed differences between C₄ and C₃ species that were driven by many metabolites, including lower ribulose 1,5-bisphosphate in C₄ species. Strikingly, there was also considerable variation between C₃ species. This was partly due to different chlorophyll and protein contents, but mainly to differences in relative levels of metabolites. Correlation analysis indicated that one contributory factor was the balance between fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, phosphoribulokinase, and Rubisco. Our results point to the CBC having experienced different evolutionary trajectories in C₃ species since the ancestors of modern plant lineages diverged. They underline the need to understand CBC operation in a wide range of species.

Keywords: C₄, C₃, Calvin–Benson cycle, interspecies variation, metabolite profiles, photosynthesis.

Introduction

The Calvin–Benson cycle (CBC) evolved ~2 billion years ago (Rasmussen et al., 2008), is the most abundant biochemical pathway on Earth in terms of nitrogen investment (Ellis, 1979; Raven, 2013), and plays a dominant role in the global carbon (C) and O₂ cycles. The CBC can be divided into three partial processes; fixation of CO₂ (ribulose-1,5-bisphosphate...
carboxylase–oxygenase) RuBisCO into a 3-C compound, 3-phosphoglycerate (3PGA), reduction of 3PGA to triose phosphate (triose-P) using ATP and NADPH from the light reactions, and a series of reactions that use triose-P to regenerate ribulose 1,5-bisphosphate (RuBP) (von Caemmerer and Farquhar, 1981; Heldt, 2005; Stitt et al., 2010; Adam, 2017). The net gain in C exits the CBC and is converted into end-products. Despite its evolutionary age, the pathway’s structure is essentially unchanged from cyanobacteria to angiosperms.

This conservation of the CBC pathway structure is remarkable. The CBC evolved in a world in which CO2 concentrations were very high and O2 concentrations were very low. Over geological time, there has been a dramatic rise in atmospheric O2 and decline in atmospheric CO2. This uncovered a side reaction with O2, which competes with CO2 as a substrate for RuBisCO, leading to the formation of 2-phosphoglycolate (2PG) (Lorimer and Andrews, 1973; Lorimer, 1981; Tcherkez et al., 2006). 2PG is recycled via an energetically wasteful process termed photorespiration that results in the loss of 0.5 CO2 per scavenged molecule of 2PG (Somerville, 2001; Heldt, 2005). In the current atmosphere with 0.04% CO2 and 21% O2, in C3 plants about every fourth reaction is with O2 instead of CO2, leading to a 20–30% decrease in the net rate of photosynthesis (Osmond, 1981; Sharkey, 1988; Long et al., 2006; Betti et al., 2016). This side reaction decreases nitrogen use efficiency, because higher amounts of protein must be invested in the photosynthetic apparatus. This includes an especially large investment in RuBisCO, which has a relatively low catalytic rate and represents up to half of leaf protein (Ellis, 1979; Betti et al., 2016). It negatively impacts water use efficiency because a higher internal CO2 concentration is required to support a given net rate of photosynthesis, which in turn requires higher stomatal conductance and higher evaporative water loss (Ort et al., 2015; Betti et al., 2016).

Cyanobacteria and eukaryotic algae possess C-concentrating mechanisms (CCMs) that accumulate CO2 in RuBisCO-containing microstructures, the carboxysome in cyanobacteria and the pyrenoid in eukaryotic algae (Badger et al., 1998; Giordano et al., 2005; Kerfeld and Melnicki, 2016; Raven et al., 2017). These microstructures were lost in plant lineages that colonized the land. A second type of CCM evolved in terrestrial plants, bicarbonate is assimilated in the dark into 4-C acids, which are decarboxylated in the light to provide CO2 for the CBC (Shameer et al., 2018). CAM evolved in at least 35 independent lineages and is found in ~6% of current terrestrial plant species (Silvera et al., 2010). Parallel evolution of C4 and CAM in many lineages underlines the strong selective pressure exerted by low CO2 in the recent geological past.

CCMs are complex traits. For example, C4 photosynthesis requires major changes in leaf development and anatomy, gene expression patterns, and the location, levels, and properties of hundreds of enzymes and transporters (Sage et al., 2012; Heckmann et al., 2013; Sage, 2017). It is likely that its evolution involved successive steps, including the development of denser venation, modification of the size and functionality of bundle sheath cells, and stepwise specialization of metabolism in the bundle sheath and mesophyll cells (McKown and Dengler, 2007; Kocacinar et al., 2008; Nelson, 2011; Sage et al., 2013; Mallmann et al., 2014). This multistep evolutionary trajectory may explain why CCMs evolved in only a relatively small fraction of terrestrial plant lineages (Heckmann, 2016).

Low CO2 will have exerted massive selective pressure on the CBC in species that did not evolve a CCM, representing ~90% of existing terrestrial plant species (Silvera et al., 2010; Sage, 2017). Pressure will also have been exerted by other environmental factors such as water availability, temperature, and nutrient availability (Raven et al., 2017). Indeed, terrestrial C3 plants exhibit substantial variation in photosynthetic rate, with large differences between annuals and perennials, and considerable differences within these groups (Evans, 1989; Wullschleger, 1993). This includes variation in photosynthetic rate between phylogenetically related species (Galmés et al., 2014b) and within species (Drier et al., 2014). Factors contributing to variation in photosynthetic rate include differences in the rate of electron transport and carboxylation (Wullschleger, 1993), leaf nitrogen content and photosynthetic nitrogen use efficiency (Field and Mooney, 1986; Evans, 1989; Hikosaka, 2010), and differing investment strategies in short-lived (deciduous) and long-lived (evergreen) leaves (Wright et al., 2004; Donovan et al., 2011).

We know relatively little about whether there is interspecific variation in the CBC in C3 plants (Lawson et al., 2012). It is well established that RuBisCO kinetics have evolved over a long geological time scale, with selectivity for CO2 rising and catalytic rate declining between cyanobacteria and higher plants (Jordan and Ogren, 1981; Badger et al., 1998; Tcherkez et al., 2006; Savir et al., 2010; Sharwood et al., 2016a, b). Intriguingly, there is also variance over shorter evolutionary time scales. RuBisCO kinetics vary between quite closely related C3 species (Yeoh et al., 1980; Galmés et al., 2014a; Prins et al., 2016). In perennial oak, ecological adaptations have been linked to specific amino acid polymorphisms in RuBisCO (Hermida-Carrera et al., 2017). RuBisCO is inhibited by RuBP and low molecular weight inhibitors that derive from catalytic
infidelities of RuBiCO or, like 2-carboxyxyarabinitol 1-phosphate, are synthesized by other enzymes (Yeoh et al., 1980; Parry et al., 2008). There is surprising diversity in the levels and dynamics of these low molecular weight inhibitors in different C₃ species (Servaites et al., 1986; Moore et al., 1993; Charlet et al., 1997; Parry et al., 2008) and, incidentally, different C₄ species (Carmo-Silva et al., 2010). CP12 is a small regulatory protein that interacts with NADP-glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH) and phosphoribulokinase (PRK) (Gontero and Maberly, 2012; López-Calcagno et al., 2014). The action of CP12 varies between C₃ species (Howard et al., 2011; López-Calcagno et al., 2014), again pointing to interspecies variation in CBC regulation.

Some of the strongest evidence that the CBC can adapt to selection or relaxation of selection in a relatively short evolutionary time comes from studies of C₄ species. Compared with C₃ species, C₄ species contain forms of RuBiCO with a lower affinity for CO₂ and faster catalytic turnover (Yeoh et al., 1980; Sage and Seemann, 1993; Kapralov et al., 2011; Galmés et al., 2014b; Sharwood et al., 2016a, b), allowing a substantial decrease in RuBiCO abundance (Long, 1999; Ghannoum et al., 2005; Sharwood et al., 2016a, b, c). Such changes are found even within the tribe Panicoideae in which C₄ photosynthesis evolved recently (Sharwood et al., 2016a).

The operation of a pathway depends on many factors, including the abundance of the participating enzymes, their kinetic properties, and the action of regulatory mechanisms on individual enzymes and sets of enzymes. It is laborious to characterize variation in all these potential factors. Analyses of steady-state metabolite levels provide a top-down strategy to search for variation in pathway operation. This is because changes in enzyme abundance, properties, or regulation will all lead to changes in the relative levels of the metabolic intermediates in a pathway.

Information about CBC intermediate levels in different C₃ species is rather sparse. Most previous studies in C₃ plants focused on RuBP (e.g. Sage and Seemann, 1993) or a handful of metabolites such as 3PGA, triose-P, and fructose 1,6-bisphosphate (FBP), and were restricted to single species (see Stitt et al., 2010 for references). A similar picture holds for C₄ plants (Stitt and Heldt, 1985; Usuda, 1987; Leegood and von Caemmerer, 1988, 1989). The reason was partly conceptual, reflecting the idea that photosynthesis is usually limited by the light reactions or RuBiCO (Farquhar et al., 1980). Subsequent work has highlighted that photosynthesis can also be limited by reactions in the remainder of the CBC (see Stitt et al., 2010 for a review), especially sedoheptulose-1,7-bisphosphatase (SBPase) (Raines et al., 2000; Lefebvre et al., 2005; Zhu et al., 2007; Ding et al., 2016; Driever et al., 2017; Simkin et al., 2017). There were also technical reasons; until ~10 years ago it was impossible to quantify many CBC intermediates routinely. This is now possible using HPLC-MS/MS (Cruz et al., 2008; Arrivault et al., 2009; Hasunuma et al., 2010; Ma et al., 2014).

In this study, we have profiled CBC intermediates in four C₄ species and five C₃ species, representing diverse plant lineages including eudicots and monocots. We used these data to address two questions. The first is whether CBC intermediates display different profiles in C₃ and C₄ species, as would be expected if the presence of a CCM allows a different mode of CBC operation. This question provides a check that expected differences in CBC operation can be detected as changes in CBC metabolite profiles. In particular, we might expect that the lower abundance of RuBiCO (see above) results in lower levels of RuBP. Furthermore, C₄ species with dimorphic chloroplasts might have enhanced levels of 3PGA and triose-P to support an intercellular shuttle that transfers energy from the mesophyll to the bundle sheath cells. The second and major question is whether there are interspecific differences between C₃ species. This would have important implications for the evolution of the CBC and the need for a better understanding of the pathway in a broader range of C₃ species, including many of our major crops.

### Materials and methods

#### Chemicals

Carbon dioxide (¹³CO₂, isotopic purity 99 atom%) was from Campro Scientific GmbH (Berlin, Germany; www.campro.eu), N₂, O₂, and unlabelled CO₂ from Air Liquide (Germany; https://industrie.airliquide.de/), and chemicals were obtained from Sigma-Aldrich (Darmstadt, Germany; www.sigmaaldrich.com), Roche Applied Science (Mannheim, Germany; lifescience.roche.com), or Merck (www.merckmillipore.com).

#### Plant growth and harvest

Nine species (of which eight were phylogenetically diverse; Supplementary Fig. S1 at JXB online) were grown as described in Supplementary Table S1. Material was harvested by cutting leaves and quenching them immediately in a bath of liquid N₂ under growth irradiance, avoiding shading.

#### Metabolite analyses

Plant material was ground to a fine powder by hand in a mortar pre-cooled with 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 (Arrivault et al., 2009). All samples were spiked with stable isotope-labelled internal standards for correction of ion suppression and other matrix effects (Arrivault et al., 2015). 3PGA gives a broad, poorly defined peak in LC-MS/MS and was therefore quantified enzymatically (Merlo et al., 1993).

#### Chlorophyll and protein

Chl a and b were extracted and quantified as in Gibon et al. (2002). Protein was extracted from 20 mg FW ground plant material in 750 µL of buffer [0.1 M Tris–HCl, pH 8, 0.2 M NaCl, 5 mM EDTA, 2% (w/v) SDS, 0.2% (v/v) β-mercaptoethanol, and protease inhibitor cocktail (P9599, Sigma, Germany)]. The suspension was mixed well, incubated (30 min, room temperature), re-mixed, centrifuged (10 min, 1500 g, 4 °C), and the supernatant collected. Supernatants were pooled from two or (Oryza sativa and Manihot esculenta) three successive extractions. Protein was quantified colorimetrically with bicinchoninic acid (BCA Protein Assay-Reducing Agent Compatible, Thermo Fisher Scientific, Germany; www.thermofisher.com) with BSA as standard.

#### Gas exchange

CO₂ assimilation was measured using the fourth fully expanded Zea mays leaf or 5-week-old Arabidopsis thaliana rosettes using an open-flow infrared gas exchange analyser system (LI-COR Inc., Lincoln, NE, USA; www.licor.com) equipped with an integrated fluorescence chamber head (LI-6400–40, 2 cm² leaf chamber for Z. mays; LI-6400-17 whole-plant Arabidopsis chamber for A. thaliana; LI-COR Inc.). CO₂ was kept at 400 µmol mol⁻¹, leaf temperature at 29 °C for Z. mays and at 20 °C for A. thaliana, and relative humidity at 65%.
Metabolite levels in Z. mays and A. thaliana at different irradiances

One potential complication of a cross-species comparison is that each species has a different light saturation response, making it difficult to standardize growth and harvest conditions across species. We grew and harvested all species at moderate and limiting irradiance, using lower irradiance for species whose photosynthesis saturates at lower light intensities (Supplementary Table S1). In addition, for Z. mays and A. thaliana, we asked whether short-term changes in irradiance lead to major changes in the metabolite profile, using an additional lower irradiance for Z. mays (Fig. 2A, covering the range from 40% to 133% of that required for half-maximal rates of photosynthesis), and a lower and a higher near-saturating irradiance for A. thaliana (Fig. 2B, covering the range from 67% to 233% of that required for half-maximal rates of photosynthesis). The metabolite profiles were not greatly altered for either species (Fig. 2C), except that higher irradiance tended to lead to a general increase in metabolite levels. Metabolite levels in a given species were strongly correlated irrespective of irradiance (r > 0.98), whereas metabolite levels were poorly correlated between species (Fig. 2D).

Participation of pools in photosynthesis

Our approach assumes that the investigated metabolites are predominantly involved in the CBC. If they are also involved in another pathway, the total content will not provide reliable information about the size of the CBC pool. Published 13C labelling kinetics validate this assumption for N. tabacum, A. thaliana, and Z. mays (Hasunuma et al., 2010; Szecowka et al., 2013; Arrivault et al., 2017); after pulses with 13CO2, all of the CBC metabolites showed a rapid rise in 13C enrichment to reach a final value of ≥80%. One exception was SBP in maize, where 13C enrichment plateaued at ~14%. We performed analogous 13CO2 labelling experiments for M. esculenta which, like Z. mays, is a subtropical species adapted to highlight conditions. We also chose M. esculenta because it has been suggested to be a C4 or C3–C4 intermediate species (Cock et al., 1987; El-Sharkawy and Cock, 1987). A subsequent study showed that M. esculenta performs C3 photosynthesis (Edwards et al., 1990; see also De Souza et al., 2017; De Souza and Long, 2018). Time-resolved 13CO2 labelling would provide a further test that M. esculenta is a C3 species.
In *M. esculenta*, CBC intermediates rose rapidly to high (>75%) $^{13}$C enrichment (Supplementary Fig. S3A; Supplementary Dataset S2) except for SBP where enrichment plateaued at ~40% and about half of the SBP remained in the unlabelled form after 60 min (Supplementary Fig. S3B). Otherwise, the labelling time series in *M. esculenta* resembled published time series for the C$_3$ plants *A. thaliana* (Szecowka *et al.*, 2013) and *N. tabacum* (Hasunuma *et al.*, 2010). In particular, labelling of 4-C acids was very slow (Supplementary Fig. S3C).

**Chlorophyll and protein**

Leaf composition varies between species (see the Introduction). This could contribute to interspecific differences in absolute
Fig. 2. CO₂ assimilation rate in *Z. mays* and *A. thaliana*, and CBC metabolite profiles in *Z. mays* and *A. thaliana* at different short-term irradiances. *Zea mays* and *A. thaliana* were grown at 550 µmol m⁻² s⁻¹ and 120 µmol m⁻² s⁻¹ irradiance, respectively. CO₂ assimilation rate in (A) *Z. mays* (*n*=10) and (B) *A. thaliana* (*n*=9). The results are shown as mean (µmol CO₂ m⁻² s⁻¹ and µmol CO₂ g FW⁻¹ h⁻¹, for *Z. mays* and *A. thaliana*, respectively) ±SD. Arrows indicate the irradiances at which leaves were sampled for metabolite analysis. (C) *Zea mays* was harvested at growth irradiance (*Zm*, medium irradiance) or after being subjected for 4 h to 160 µmol m⁻² s⁻¹ (*ZmL*, low irradiance) from the beginning of the light period. *Arabidopsis thaliana* was harvested at growth irradiance (*At*, medium irradiance) or subjected for 15 min to 80 µmol m⁻² s⁻¹ or 280 µmol m⁻² s⁻¹ (*AtL*, low and *AtH*, high irradiances, respectively). Quenching of metabolism and harvest of leaf tissue were performed at least 4 h after the beginning of the light period. 2PG amounts are multiplied by 10 for better visibility. Asterisks indicate graphs already presented in Fig. 1. The results are shown as mean (nmol g FW⁻¹) ±SD. (D) Correlation analysis. The metabolite data shown in (B) and (C) were used to perform Pearson’s correlation analysis between data sets from the same species at different irradiances, and correlations between different species. Before performing the correlation analysis, each data set was normalized by calculating the amount of carbon in a given metabolite, and dividing it by the total carbon in all metabolites in that data set. This was done to avoid secondary correlation due to any interspecies differences in leaf composition. The results are given as *r* and the higher correlations are indicated in bold. All correlations were positive. The original data are presented in Supplementary Dataset S1.
metabolite levels; in particular, differences in leaf composition could lead to systematically higher or lower levels of all metabolites. We therefore determined total chlorophyll and protein contents in the leaf material used for metabolite analyses. Total chlorophyll content (Fig. 3A) was similar on a FW basis in all species except for O. sativa and M. esculenta, which had considerably higher values. Protein content on a FW basis (Fig. 3B) was similar in all species except for lower values in N. tabacum, and higher values in O. sativa and, especially, M. esculenta. These results partly explain why CBC metabolite levels on a FW basis tended to be low in N. tabacum and high in O. sativa and M. esculenta (Fig. 1).

Principal component analysis

We performed principal component (PC) analyses to provide an integrated overview of the CBC metabolite profiles in the nine species. PC analysis gives information about which samples (here, different species) are closely related or separated, and which variables (here, metabolites) contribute to this relationship. The analysis was performed with z-scored data (i.e. normalizing the individual values of a given variable on the mean value for that variable) to ensure that each metabolite adopted an equally important role in the analysis, independent of its absolute abundance. Each individual sample was included separately in the analysis to provide an overview of the quality of within-species replication. We included the low light maize and the low and high light Arabidopsis samples to further test the impact of prevailing irradiance. In the analyses shown in Fig. 4, we omitted 2PG to focus solely on the CBC and exclude effects due to lower photosynthesis in C4 plants. We also omitted SBP because of the labelling data (Supplementary Fig. S3; Arrivault et al., 2017) indicating that in some species part of the SBP pool is not involved in the CBC. For comparison, analyses including 2PG and SBP are provided in Supplementary Figs S4–S7.

As previously mentioned, some cross-species variation in metabolite levels may be driven by changes in leaf composition. We therefore performed PC analyses on data sets in which the metabolites were normalized on FW (Fig. 4A; Supplementary Fig. S4), total chlorophyll content (Fig. 4B; Supplementary Fig. S5), or protein content (Fig. 4C; Supplementary Fig. S6). We also performed PC analysis on a dimensionless data set in which, for a given species, the amount of C in a given metabolite was divided by the total amount of C in all CBC intermediates plus 2PG (Fig. 4D; Supplementary Fig. S7). In total, we performed 16 PC analyses with different metabolite data sets and normalizations. In interpreting the plots, we focused on features that were seen in all or the vast majority of these analyses.

In analyses with the FW-, chlorophyll-, and protein-normalized data sets and the dimensionless data set, PC1 accounted for 44–46, 33–35, 34–36, and 39–40%, respectively, of the total variance, while PC2 accounted for 17–20, 20–23, 21–22, and 19–22%, respectively (Fig. 4A–C; Supplementary Figs S4–S6). In all cases, replicates for a given species grouped together, showing that within-species variance was smaller than inter-species differences. This included samples harvested at low and ambient light intensities for Z. mays and for A. thaliana. The A. thaliana samples collected at high light grouped separately from the other A. thaliana samples, but well removed from the other species in PC analyses with the FW-, chlorophyll-, and protein-normalized data sets. In PC analyses with the dimensionless data set, A. thaliana samples from all three light intensities grouped together (Fig. 4D; Supplementary Fig. S7), showing that increasing light intensity led mainly to a general increase in metabolite levels rather than to changes in their relative levels.

In all analyses, samples for a given species fell into one of three main groups. First, the PC analyses almost always separated C3 species from C4 species; this holds irrespective of how the metabolite data are normalized, and whether 2PG and SBP were excluded (Fig. 4) or included (Supplementary Figs S4–S7). Manihot esculenta showed a slight overlap with the Flaveria spp. in the analyses using metabolites minus SBP and 2PG, when the data set was normalized on protein (Fig. 4C), but was fully separated from all of the C4 species in the 15 other PC analyses. A. thaliana in low light showed a slight overlap with Z. mays or S. viridis in two (all metabolites normalized on FW; metabolites minus SBP normalized on FW; Supplementary
Data are presented in Supplementary Dataset S1. On total chlorophyll content, Supplementary Fig. S6 (amounts normalized on protein content), and Supplementary Fig. S7 (dimensionless). The original metabolites except either 2PG or SBP are shown in Supplementary Fig. S4 (amounts normalized on FW), Supplementary Fig. S5 (amounts normalized on total chlorophyll). The loadings of CBC intermediates in PC1 and PC2 are shown in red. Principal component analyses with the full metabolite data set and with all metabolites except either 2PG or SBP are shown in Supplementary Fig. S4 (amounts normalized on FW), Supplementary Fig. S5 (amounts normalized on total chlorophyll content), Supplementary Fig. S6 (amounts normalized on protein content), and Supplementary Fig. S7 (dimensionless). The original data are presented in Supplementary Dataset S1. This transformation generates a dimensionless data set (provided in Supplementary Dataset S1) in which each metabolite receives a value equal to its fractional contribution to all the C in CBC metabolites plus 2PG. As this data set is dimensionless, there is no systematic bias due to differences in leaf composition. The distribution of C₄ species (green) and C₃ species (black) is shown on PC1 and PC2 (Fig. 4). The loadings of CBC intermediates in PC1 and PC2 are shown in red. Principal component analyses with the full metabolite data set and with all metabolites except either 2PG or SBP are shown in Supplementary Fig. S4 (amounts normalized on FW), Supplementary Fig. S5 (amounts normalized on total chlorophyll content), Supplementary Fig. S6 (amounts normalized on protein content), and Supplementary Fig. S7 (dimensionless). The original data are presented in Supplementary Dataset S1.

Fig. S4) of the 16 data permutations. Secondly, within the C₄ species, Z. mays and S. viridis separated from each other and from the Flaveria spp. in most of the PC analyses, while the two Flaveria spp. always overlapped with each other. Thirdly, the five C₃ species were almost always clearly separated from each other. In the analyses based on FW-normalized data, O. sativa
and *M. esculenta* separated strongly from other C3 species in PC1 (Fig. 4A; Supplementary Fig. S4). This was less marked in the PC analysis based on chlorophyll- or protein-normalized data (Fig. 4B, C; Supplementary Figs S5, S6), indicating that the strong separation in the analysis with FW-normalized data is partly driven by secondary effects due to leaf composition. Similarly, *N. tabacum* was less strongly separated from the other four C3 species in the PC analysis with protein-normalized data than with FW- or chlorophyll-normalized data. Despite these small shifts in the relationships, the five C3 species still separated from each other in the PC analyses with the chlorophyll- and protein-normalized data sets, as well as with the dimensionless data set (Fig. 4D; Supplementary Fig. S7).

The metabolite loadings (Fig. 4; Supplementary Figs S4–S7) reveal that the separation of C4 from C3 species was driven not only by lower levels of RuBP and (when included) 2PG, but also by other CBC metabolites. 3PGA and triose-P contributed to the separation of the C4 species *Z. mays* and *S. viridis* from *F. trinervia* and *F. bidentis* (see the Discussion). Almost every metabolite contributed to the separation between the five C3 species, with large contributions from RuBP, FBP, F6P, S7P, ribose 5-phosphate (R5P), triose-P, and 3PGA.

We repeated the PC analysis on a data set including only C3 species and with metabolites normalized on total chlorophyll content or protein content, and with a dimensionless data set (Fig. 5; Supplementary Figs S8–S10). Replicate samples from a given species grouped closely together. *A. thaliana*, *N. tabacum*, and *M. esculenta* were clearly separated from *T. aestivum* and *O. sativa*, which were only weakly separated. The high irradiance *A. thaliana* samples grouped separately from the low and medium light *A. thaliana* samples, but in the same tangent, and were clearly separated from the other four C3 species. Metabolite loadings revealed strong contributions from 3PGA, triose-P, RuBP, FBP, F6P, and S7P to the separation.

**Coefficient of variance**

We calculated the coefficient of variance (CV) to determine which metabolites showed the greatest interspecies variance for all nine species together, for the four C4 species, and for the five C3 species (Fig. 6). To avoid influence due to leaf composition, this analysis was performed on the dimensionless data set. Across all species (Fig. 6A), the highest CV was for 2PG, followed by FBP, RuBP, triose-P, SBP, and R5P. When only C4 species are considered (Fig. 6B), the highest CV was for SBP, followed by 2PG, RuBP, R5P, FBP, and triose-P. When only C3 species are considered (Fig. 6C), the highest CV was for FBP, followed by Ru5P+Xu5P, 2PG, R5P, and 3PGA.

**Correlation analysis**

When metabolite profiles are compared across different genotypes, they typically generate a correlation network (Meyer et al., 2007; Sulcipe et al., 2009, 2013; Zhang et al., 2015; Wu et al., 2016). This reflects features of the underlying metabolic pathways that are maintained across genotypes and generate conserved relationships between metabolites. Our data set allowed us to apply this approach to interspecies variation in the CBC.
The CBC correlation network for all nine species (Fig. 7A) contained six positive correlations (e.g. F6P versus S7P; all pairwise comparisons between RuBP, FBP, and SBP), many non-significant relationships [e.g. FBP versus F6P; SBP versus S7P; RuBP versus R5P and Ru5P+Xu5P (here collectively called pentose-P)], and 13 negative correlations (e.g. 3PGA or triose-P versus most other CBC metabolites). In some cases, the correlations were driven by differences between C4 and C3 species; for example, the positive correlation between 2PG and RuBP is driven by the lower levels of both metabolites in C4 compared with C3 species (see Fig. 1). However, in many cases, the correlations were also seen within the subset of C4 and within the subset of C3 species (see Supplementary Fig. S11 and below).

The correlation network for CBC metabolites in C4 species (Fig. 7B) contained nine positive (e.g. FBP versus S7P; FBP versus RuBP; and triose-P versus FBP, SBP, and RuBP) and 13 negative (e.g. 3PGA versus triose-P, FBP, and SBP; triose-P versus S7P and pentose-P; RuBP versus F6P, S7P, and pentose-P; and SBP versus S7P) relationships. There was no significant relationship between FBP and F6P. The correlation network for CBC metabolites in C3 species (Fig. 7C) contained six positive (e.g. all pairwise comparisons between RuBP, FBP, and SBP; and F6P versus S7P) and 11 negative (e.g. 3PGA versus triose-P, FBP, SBP, and RuBP; RuBP versus F6P and S7P; and SBP versus S7P) relationships. There was no significant relationship between FBP and F6P, or between RuBP and pentose-P. 2PG correlated positively with S7P and negatively with RuBP in C4 and C3 species, respectively, and positively with 3PGA and negatively with triose-P and FBP in C3 species.

The correlation networks can be interpreted by relating them to CBC topology (Fig. 7D; see also Supplementary Fig. S11). Figure 7D focuses on correlations seen within the subset of C4 species and within the subset of C3 species. Triose-Ps are used to synthesize FBP and SBP in reversible reactions catalysed by aldolase. This may explain the positive correlations between triose-P and FBP or SBP (except for SBP in C3 plants). FBPase and SBPase catalyse irreversible reactions. The non-significant or negative correlations between FBP and F6P and between SBP and S7P point to interspecies differences in the regulation of FBPase and SBPase. This may also explain the absence of a positive correlation between triose-P and pentose-P that otherwise might have been expected because pentose-Ps are formed from triose-P and F6P or S7P in reversible reactions catalysed by transketolase (TK). The negative relationship between pentose-P and RuBP points to interspecies variation in the regulation of PRK. Further, the positive correlations of FBP and SBP with RuBP (see Supplementary Fig. S11) indicate that FBPase and SBPase activity vary reciprocally to PRK activity and/or co-ordinately with binding or use of RuBP by RubisCO.

**Discussion**

The CBC is an ancient pathway that has been under selective pressure due to the long-term increase of the O2/CO2 ratio in the atmosphere and particularly over the last 30 million years...
Metabolite profiles reveal diversity in the Calvin–Benson cycle

Fig. 7. Correlation between levels of CBC metabolites. The correlations were performed with all individual samples from a dimensionless data set, generated as described for Fig. 4D. The transformed data were used to calculate the Pearson’s correlation matrix on every pair of metabolites. Correlation values are given in the figure panels and indicated by a heat map. The adjacent dendrograms show clusters defined using the complete linkage method (Sørensen, 1948). Non-significant correlations ($P ≥ 0.05$; two-sided Student’s $t$-test) are set as zero. Metabolite pairs that are linked by irreversible reactions are indicated by a black box. (A) All species, (B) only C4 species, and (C) only C3 species. An alternative display is provided in Supplementary Fig. S11, with the same fixed order of metabolites in each panel, corresponding to the reaction sequence in the CBC. The same heat map scale is used for (A–C). (D) Schematic representation of interspecies variance in the ratio of substrate abundance:product abundance for different CBC enzymes. Enzymes that catalyse irreversible reactions are highlighted in bold. For each enzyme reaction, the substrate and product that were compared are indicated in the list below the display. This display is schematic because some metabolites were not measured (erythrose 4-phosphate, E4P; and glyceraldehyde 3-phosphate, GAP) or were not separated (Ru5P and Xu5P). For reactions using GAP, it is assumed that GAP and dihydroxyacetone phosphate (DHAP) are in equilibrium. For transketolase (TK), two reactions were separated (termed TKa and TKb). For TKa, the reactant E4P was missing, and only the relationships between triose-P and F6P and Ru5P-Xu5P are shown. For TKb, the plot focuses on the relationship between S7P and R5P or Ru5P+Xu5P. The display shows the alternative pairs of metabolites compared, with the upper and lower symbols in the display corresponding to the upper and lower pair in the list. A similar display mode is used for the carboxylation and oxygenation reactions of Rubisco. The correlation coefficients are taken from (B) and (C), using the same heat map scale. Results are shown separated for correlations between the four C4 species (squares) and the five C3 species (circles). The analysis is not shown for the combined C4 plus C3 species set because, in this case, some relationships are driven by differences between C4 and C3 species. Additional abbreviations: fructose 1,6-bisphosphate aldolase (FBP ald), phosphoglycerate kinase (PGK), ribose 5-phosphate isomerase (RSP isom), sedoheptulose 1,7-bisphosphate aldolase (SBP ald).
due to falling CO₂ concentrations, which led to independent evolution of a CCM in >100 terrestrial plant lineages. However, the vast majority of terrestrial species did not evolve a CCM, probably because they were unable to follow the multistep evolutionary trajectory that was required to acquire this complex trait (Sage et al., 2012; Christin and Osborne, 2013; Heckmann et al., 2013). Present-day C₃ plants nevertheless will have been subject to similar selective pressures to those that drove the evolution of C₄ or CAM photosynthesis. Indeed, in the absence of a CCM, the selective pressures on the CBC may have been even greater than in plants that did evolve a CCM. In addition to low CO₂, it is likely that environmental factors such as irradiance, temperature, and nutrient and water availability exerted more or less selective pressure, depending on the local environment, and leading to different evolutionary trajectories in different populations. While it is well documented that there is large variation in photosynthetic rate between terrestrial species (Evans, 1989; Wullschleger, 1993; Lawson et al., 2012), previous studies of the underlying causes have focused on leaf morphology and composition (Field and Mooney, 1986; Evans, 1989; Hikosaka, 2010; Poorter et al., 2015; Diaz et al., 2016), stomatal conductance (Lawson et al., 2012), and the kinetic characteristics of RuBisCO (Yeoh et al., 1980; Jordan and Ogren, 1981; Badger et al., 1998; Tcherkez et al., 2006; Galmés et al., 2014b; Prins et al., 2016; Sharwood et al., 2016a, b). Little is known about whether the CBC operates in a highly conserved manner or in different modes in different C₃ species.

We have used metabolite profiling as an unbiased strategy to search for interspecific variance in CBC operation. The underlying assumption is that changes in the balance between different enzymatic steps will lead to changes in the relative levels of pathway intermediates. This approach is top down, in the sense that it does not make assumptions about whether the observed variance is due to changes in gene expression and protein abundance, enzyme kinetics, or regulatory networks that act on the enzymes. We applied it to search for differences in CBC operation between C₄ and C₃ plants, and within C₃ species. As our aim was to compare CBC operation across species, we focused exclusively on the metabolites that are involved in the CBC plus 2PG, the immediate product of the RuBisCO oxygenation reaction. We excluded metabolites involved further downstream in photorespiration and metabolites involved in the CO₂-concentrating shuttle in C₄ plants, which have non-photosynthetic functions in C₃ plants.

Our interspecies comparison required important control experiments and cross-checks during data analysis. First, plant species differ in their photosynthetic rate and its dependence on light, temperature, and the availability of water, nutrients, and CO₂ (see the Introduction). We grew and harvested plants in a light regime that was limiting for that species, rather than using identical conditions for all species. In these conditions, RuBP regeneration is likely to be limiting, and effects of light stress are avoided. Importantly, we showed for one C₄ species (Z. mays) and one C₃ species (A. thaliana) that although increased harvest irradiance led to higher levels of metabolites, it did not strongly alter their relative levels (Fig. 2). Secondly, it is important that the CBC pool accounts for most or all of the total content of a given metabolite. Analysis of published data for two C₃ (N. tabacum and A. thaliana), one C₄ (Z. mays) species (Hasunuma et al., 2010; Szecowka et al., 2013; Arrivault et al., 2017), and a new data set for the C₃ species M. esculenta (Supplementary Fig. S3) showed that CBC intermediates exhibit a rapid rise in ¹³C enrichment to a high level after supplying ¹³CO₂. This provides evidence that most of the total pool is indeed involved in the CBC. This conclusion is supported by published subcellular fractionation studies, in which most CBC intermediates are exclusively or largely confined to the plastid (Gerhardt et al., 1987; Szecowka et al., 2013). The only exception was SBP, which was only partially labelled in Z. mays and M. esculenta. We do not know whether there is a separate pool of SBP that is not involved in CO₂ fixation, or if these plant species contain an unknown metabolite with an identical chromatographic behaviour, mass, and fragmentation pattern to SBP. In our interpretation of the metabolite profiles, we took care that our conclusions did not depend on inclusion of SBP. A third set of controls addressed the issue that leaf composition varies between species, with the result that absolute values for metabolite content will depend on the unit in which they are given. We analysed metabolite data normalized on FW, chlorophyll, or protein content, and also used a dimensionless data set in which metabolite levels were expressed relative to each other. Our interpretation focused on results that were independent of how the data were normalized. Importantly, inclusion of the dimensionless data set eliminated secondary correlations due to differences in leaf composition, and placed the emphasis on relative rather than absolute levels of metabolites. It minimizes contributions from differing light regimes, which had less effect on relative than on absolute metabolite levels (see above).

We included four C₄ species in our panel to test if CBC profiles could distinguish between species in which it is known that the CBC operates in a different context from that of C₃ plants. The CBC operates at a much higher intercellular CO₂ concentration in C₄ than in C₃ plants, and RuBisCO has a higher affinity for CO₂, and an increased catalytic rate in C₄ compared with C₃ species (see the Introduction). PC analysis confirmed that CBC metabolite profiles allow C₄ and C₃ species to be distinguished (Fig. 4; Supplementary Figs S4–S7). As expected, C₄ species had lower 2PG and RuBP than C₃ species (Fig. 1). However, the separation in the PC analysis was also seen when 2PG was excluded, and was driven by several other CBC intermediates, pointing to broader changes in CBC operation between C₄ and C₃ species.

The four C₄ species belong to the NADP-malic enzyme C₄ subtype. Interestingly, PC analysis separated Z. mays and S. viridis from the two Flaveria spp. Whilst this might reflect a difference between monocots and eudicots, the PC vectors indicated that this separation reflected higher levels of 3PGA and, in particular, triose-P in Z. mays and S. viridis (Fig. 4; Supplementary Figs S4–S7; see also Fig. 1). Most NADP-malic enzyme C₄ subtypes, including Z. mays, have dimorphic chloroplasts with little or no PSII activity in the bundle sheath cells (Muneke, 2016). They operate an intercellular shuttle in which 3PGA moves from the bundle sheath to the mesophyll cells and is reduced to triose-P, which returns to the
bundle sheath. Intercellular movement is thought to occur by diffusion (Hatch and Osmond, 1976), driven by concentration gradients that require the build-up of large pools of 3PGA and triose-P in the bundle sheath and mesophyll cells, respectively (Leegood, 1985; Stitt and Heldt, 1985; Arrivault et al., 2017). *Flaveria bidentis* and *F. trinervia* can have PSII activity in the bundle sheath chloroplasts, although to a varying extent depending on conditions (Laetsch and Price, 1969; Höfer et al., 1992; Meister et al., 1996; Nakamura et al., 2013). Their separa-
tion in the PC analysis from *Z. mays* and *S. viridis* might reflect
decreased reliance on this intercellular shuttle.

Our panel included five *C*₃ species, two monocots (*O. sativa* and *T. aestivum*) and three eudicots (*A. thaliana*, *N. tabacum*, and *M. esculenta*), with the individual species representing different phylogenetic lineages (Supplementary Fig. S1) and originating in differing climatic zones. The three *C*₃ eudicot species represent two of the major lineages within the eudicots, namely the asterids (*N. tabacum*) and rosids (*A. thaliana* and *M. esculenta*), that contain 41% and 24% of all angiosperms, respectively. There was considerable interspecies variation in CBC metabolite profiles. This was evident from visual inspection of the metabolite levels (Fig. 1) and was confirmed by PC (Figs 4, 5; Supplementary Figs S4–S10) and variance (Fig. 6) analyses.

When metabolites were expressed on a FW basis, some of the variation was due to differences in leaf composition, with a strong trend to higher absolute levels in *O. sativa* and *M. esculenta*, reflecting their high chlorophyll and protein content. The high protein content in *O. sativa* may be linked to changes in leaf anatomy that enhance mesophyll transfer conductance, including small deeply lobed cells and densely arranged chloroplasts and stomata at the cell surface (Sage and Sage, 2009; Busch et al., 2013). This high mesophyll transfer conductance may prevent internal CO₂ from being drawn down by the high CBC activity that results from the high protein and metabolite content per unit FW in *O. sativa*. The high protein content in *M. esculenta* resembles the findings of previous reports (Awoyinka et al., 1995; Nassar and Marques, 2006), and could explain the high rates of photosynthesis in this species.

However, the five *C*₃ species still showed differing CBC metabolite profiles when metabolites were expressed on a chlorophyll or protein basis, or when the analyses were performed with a dimensionless data set. Variation was driven by many metabolites including RuBP, 3PGA, triose-P, FBP, F6P, S7P, and Ru5P+Xu5P. This variation points to different operating modes of the CBC in different *C*₃ species. There were also differences in 2PG content; this might be related to the rate of RuBisCO oxygenation or removal of 2PG by 2-phosphoglycolate phosphatase.

Cross-species correlation analysis (Fig. 7; Supplementary Fig. S11) revealed that in both *C*₄ and *C*₃ species, the inter-
species variance often included parallel changes of FBP, SBP, and RuBP, and unrelated or even reciprocal changes of these metabolites to F6P, S7P, and pentose-P. This is consistent with interspecies variation in the balance between FBPase, SBPase, PRK, and RuBisCO activity. It could reflect differences in the abundance or the regulation of these enzymes, both within *C*₃ species and within *C*₄ species, and between *C*₃ and *C*₄ spe-
cies. Little is known about the expression, characteristics, and
regulation of CBC enzymes in different species, with (see the Introduction) the exception of RuBisCO.

Our results do not reveal when and under what circumstances the variation in CBC function in *C*₃ species appeared. It is tempting to link it with the selection pressure that led to the appearance of *C*₄ and CAM photosynthesis, but it is likely to have started even earlier. Further, as pointed out by Zhu et al. (2007), it is possible that different *C*₃ species are following different trajectories during the increase in CO₂ levels in very recent evolutionary time. Our results also indicate that there is no strong connection between phylogeny and the diversity in CBC metabolite profiles in *C*₃ species. In the PC analyses (Figs 4, 5; Supplementary Figs S4–S10), the two monocot species are often closely related, but the three eudicot species are highly diverse, and a given eudicot is often more closely related to the monocot species than to the other eudicot species. Unlike changes in genome sequence, complex emergent phenotypes may not accrue in a linear manner, and phylogenetically dis-
tinct species may undergo convergent evolution whilst phylo-
genetically related species may undergo divergent evolution, depending on the selective pressure they experience. Better understanding of the relationship between diversity in CBC profile, phylogeny, and evolution will require studies both with more phylogenetically diverse species and with more dense sampling in short evolutionary space.

In conclusion, marked differences in CBC metabolite profiles between five *C*₃ species, including the major crop plants *O. sativa*, *T. aestivum*, and *M. esculenta*, and the important model plants *A. thaliana* and *N. tabacum*, reveal interspecies variation in the operating mode of the CBC in *C*₃ plants. This probably reflects independent evolution of CBC regulation in different plant lineages, in analogy to the independent evo-
lution of a CCM in different plant lineages. These findings, together with emerging evidence for interspecies variation in the properties of specific CBC enzymes (see the Introduction) and the growing realization that efficient photosynthesis requires integrated operation of the CBC (Stitt et al., 2010; Raines, 2011; Simkin et al., 2017), highlight the need for a mechanistic understanding of CBC regulation in a wider range of species. This will be an important step towards improving *C*₃ photosynthesis and crop productivity.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Phylogenetic distribution based on APGIII of the tested plant species.

Fig. S2. Experimental set-up for 13CO₂ labelling of *M. esculenta*.

Fig. S3. 13C enrichment (%) of CBC metabolites, relative abundance (%) of SBP isotopomers, and 13C enrichment (%) of malate, aspartate, pyruvate, and alanine in *M. esculenta*.

Fig. S4. PC analyses of all species using metabolite data nor-
malized on FW (Supplementary analyses to Fig. 4A).

Fig. S5. PC analyses on all species using metabolite data nor-
malized on total chlorophyll content (supplementary analyses to Fig. 4B).
Fig. S6. PC analyses on all species using metabolite data normalized on protein content (supplementary analyses to Fig. 4C).

Fig. S7. PC analyses on all species using a dimensionless data set (supplementary analyses to Fig. 4D).

Fig. S8. PC analyses on C3 species only, using metabolite data normalized on total chlorophyll content (supplementary analyses to Fig. 5A).

Fig. S9. PC analyses on C3 species only, using metabolite data normalized on protein content (supplementary analyses to Fig. 5B).

Fig. S10. PC analyses on C3 species only, using a dimensionless data set (supplementary analyses to Fig. 5C).

Fig. S11. Correlation between levels of CBC metabolites, with metabolites shown in a fixed order reflecting the reaction sequence in the CBC.

Table S1. Growth conditions and photosynthetic rates.
Dataset S1. Metabolite levels, total chlorophyll, and protein contents in different species.
Dataset S2. Labelling kinetics of CBC and other intermediates after exposing M. esculenta to 13CO2 (supplementary data to Supplementary Fig. S3).

Acknowledgements

This research was supported by the Max Planck Society (TAM, AR, FAB, JEL, AS, MG, and MS), the Bill and Melinda Gates Foundation (CASS to SA and TO; C2, Rice to GLB), the German Ministry of Education and Research (FullThrottle, grant 03IB0205A to DBM), CNPq (to TAM), and the Australian Research Council (to ML, JEL, and MS). We thank Christin Abel, Ina Krahert, and Dr. Mark Aurel Schöttler for help with plant growth.

References

Adam NR. 2017. C3 carbon reduction cycle: eLS. Chichester: John Wiley & Sons, Ltd.
Arrivault S, Guenther M, Fry SC, Fuenfgeld MM, Veyel D, Mettler-Altmann 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, 6986–6994.
Arrivault S, Guenther M, Ivakov A, Feil R, Vosloh D, van Dongen JT, Sulpice 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 rosettes at different carbon dioxide concentrations. The Plant Journal 59, 609–621.
Arrivault S, Obata T, Sulpice R, Stitt M. 2008. Oligoglena CO2 decline promoted C4 photosynthesis in grasses. Current Biology 18, 37–43.
Charlton T, Moore BD, Seemann JR. 1997. Carboxyarabinitol 1-phosphate phosphatase from leaves of Phaseolus vulgaris and other species. Plant & Cell Physiology 38, 511–517.
Christin PA, Besnard G, Samaritani E, Duvall MR, Hodkinson TR, Savolainen V, Salamin N. 2008. Oligoglena CO2 decline promoted C4 photosynthesis in grasses. Current Biology 18, 37–43.
Charlton PA, Osborne CP. 2013. The recurrent assembly of C4 photosynthesis, an evolutionary tale. Photosynthesis Research 117, 163–175.
Cock JH, Riaño NM, El-Sharkawy MA, Yamel LF, Bastidas G. 1987. C3-C4 intermediate photosynthetic characteristics of cassava (Manihot esculenta Crantz): II. Initial products of 14CO2 fixation. Photosynthesis Research 12, 237–241.
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.
De Souza AP, Long SP. 2018. Toward improving photosynthesis in cassava: characterizing photosynthetic limitations in four current African cultivars. Food and Energy Security 7, e00130.
De Souza AP, Massenburg LN, Jaiswal D, Cheng S, Shekar R, Long SP. 2017. Rooting for cassava: insights into photosynthesis and associated physiology as a route to improve yield potential. New Phytologist 213, 50–55.
Diaz S, Katte J, Cornelissen JH, et al. 2016. The global spectrum of plant form and function. Nature 539, 167–171.
Ding F, Wang M, Zhang S, Ai X. 2016. Changes in SBPase activity influence photosynthetic capacity, growth, and tolerance to chilling stress in transgenic tomato plants. Scientific Reports 6, 32741.
Donovan LA, Maherali H, Garuso CM, Huber H, de Kroon H. 2011. The evolution of the worldwide leaf economics spectrum. Trends in Ecology & Evolution 26, 88–95.
Driever SM, Lawson T, ArahaloPJ, Raines CA, Parry MA. 2014. Natural variation in photosynthetic capacity, growth, and yield in field-grown wheat genotypes. Journal of Experimental Botany 65, 4959–4973.
Driever SM, Simkin AJ, Aloitaib S, et al. 2017. Increased SBPase activity improves photosynthesis and grain yield in wheat grown in greenhouse conditions. Philosophical Transactions of the Royal Society B: Biological Sciences 372, 1730.
Edwards EJ, Osborne C, Strömberg CA, et al. 2010. The origins of C4 grasslands: integrating evolutionary and ecosystem science. Science 328, 587–591.
Edwards GE, Sheta E, Moore BD, Dai Z, Frario-echi VR, Cheng S-H, Lin C-H, Ku MSB. 1990. Photosynthetic characteristics of cassava (Manihot esculenta Crantz), a C4 species with chlorophylls bundle sheath cells. Plant & Cell Physiology 31, 1199–1206.
El-Sharkawy MA, Cock JH. 1987. C3-C4 intermediate photosynthetic characteristics of cassava (Manihot esculenta Crantz): I. Gas exchange. Photosynthesis Research 12, 219–235.
Ellis RJ. 1979. The most abundant protein in the world. Trends in Biochemical Sciences 4, 241–244.
Evans JR. 1989. Photosynthesis and nitrogen relationships in leaves of C3 plants. Oecologia 78, 9–19.
Farquhar GD, von Caemmerer S, Berry JA. 1980. A biochemical model of photosynthetic CO2 assimilation in leaves of C3 species. Planta 149, 78–90.
Field C, Mooney HA. 1986. The photosynthesis–nitrogen relationship in wild plants. In: Givnish T, ed. On the economy of plant form and function. Cambridge: Cambridge University Press, 25–55.
Galmés J, ArahaloPJ, Kapralov MV, Flexas J, Keys AJ, Molinos A, Parry MA, Conesa MA. 2014a. Environmentally driven evolution of Rubisco and improved photosynthesis and growth within the C4 genus Limonium (Plumbaginaceae). New Phytologist 203, 989–999.
Temperature responses of Rubisco from Paniceae grasses provide further evidence of their high photosynthetic efficiency, as seen through the non-cyclic photophosphorylation process. This highlights the importance of Rubisco in maintaining the balance between carbon gain and oxygen evolution. Additionally, the optimization of Rubisco catalytic activity and adaptation toward optimality in a low-dimensional landscape is crucial for plant survival under different environmental conditions. Cross-species analysis tracing the evolution of Rubisco provides insights into the genetic and evolutionary forces driving the specificity for refixation of photorespiratory CO2 and efforts to engineer C4 photosynthesis, which is essential for improving wheat photosynthesis and reducing the impact of climate change. Improving analysis of C4 and C3 photosynthesis via refined models is critical for understanding the adaptability and plasticity of different plant species in response to environmental changes.