Title:

A leaf–level biochemical model simulating the introduction of C\(_2\) and C\(_4\) photosynthesis in C\(_3\) rice: gains, losses and metabolite fluxes

Authors and addresses:

Chandra Bellasio\(^1\)-\(^3\)* and Graham D Farquhar\(^1\)

\(^1\)Research School of Biology, Australian National University, Acton, ACT, 2601 Australia;
\(^2\)University of the Balearic Islands 07122 Palma, Illes Balears, Spain;
\(^3\)Trees and Timber institute, National Research Council of Italy, 50019 Sesto Fiorentino (Florence).

Correspondence details:

chandra.bellasio@anu.edu.au

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File 2: The model, coded in Excel, made freely available.
A leaf–level biochemical model simulating the introduction of C2 and C4 photosynthesis in C3 rice: gains, losses and metabolite fluxes

Chandra Bellasio1–3* and Graham D Farquhar1

1Research School of Biology, Australian National University, Acton, ACT, 2601 Australia;
2University of the Balearic Islands 07122 Palma, Illes Balears, Spain;
3Trees and Timber institute, National Research Council of Italy, 50019 Sesto Fiorentino (Florence).

*Correspondence: chandra.bellasio@anu.edu.au

Summary:

• This work aims at developing an adequate theoretical basis for comparing assimilation of the ancestral C3 pathway with CO2 concentrating mechanisms (CCM) that have evolved to reduce photorespiratory yield losses.

• We present a novel model for C3, C2, C2+C4 and C4 photosynthesis simulating assimilatory metabolism, energetics, and metabolite traffic at the leaf–level. It integrates a mechanistic description of light reactions to simulate ATP and NADPH production, and a variable engagement of cyclic electron flow. The analytical solutions are compact and thus suitable for larger scale simulations. Inputs were derived with a comprehensive gas exchange experiment.

• We show trade–offs in the operation of C4 that are in line with ecophysiological data. C4 has the potential to increase assimilation over C3 at high temperatures and light intensities, but this benefit is reversed under low temperatures and light.

• We apply the model to simulating the introduction of progressively complex levels of CCM into C3 rice, which feeds more than 3.5 billion people. Increasing assimilation will require considerable modifications such as expressing the NDH complex and upregulating cyclic electron flow, enlarging the bundle sheath, and expressing suitable transporters to allow adequate metabolite traffic. The simpler C2 rice may be a desirable alternative.

Keywords


Running title

Simulating biochemical carbon concentrating mechanisms
Introduction

Carbon concentrating mechanisms (CCM; acronyms are listed in Table 1) are co–ordinated suites of structural and biochemical modifications to ancestral C3 photosynthesis. CCMs evolved to reduce the magnitude of photorespiration, a complex process resulting in the release of previously fixed CO2, which incurs substantial energy costs to recycle by–products (Meyer & Griffiths, 2013). In plants, CCMs have the form of biochemical cycles that increase the CO2/O2 ratio at the Rubisco catalytic site, and are of two types: the ‘C2 shuttle’ and the C4 cycle. To operate a CCM, the photosynthetic parenchyma is often differentiated into two cell types, although single–celled systems do exist (King et al., 2012): an external layer of mesophyll (M) and an internal layer of bundle sheath (BS) encircling the vasculature (Lundgren et al., 2014). The C2 shuttle consists of the compartmentation of glycine decarboxylase (GDC) activity in the BS, delivering CO2 around Rubisco in the BS, using the photorespiratory glycine produced in the M (Keerberg et al., 2014). The C4 cycle represents a further sophistication involving an energy dependent carboxylation–decarboxylation cycle. CO2 is initially fixed into a four–carbon (C4) organic acid (OAA) in the M by phosphoenolpyruvate carboxylase (PEPC), which after reduction (or transamination) diffuses to the BS where it is decarboxylated. If, on the one hand, the C4 cycle lowers the photorespiratory ATP demand, on the other it requires a considerable amount of ATP (2 ATP per CO2 pumped, for the NADP–ME subtype) for the regeneration of phosphoenolpyruvate (Kanai & Edwards, 1999; Evans et al., 2007; Bellasio, 2017). In ‘C2+C4’ species (Bellasio, 2017) the degree of PEPC engagement, and the extent of Rubisco compartmentation to the BS are intermediate and are species dependent (Monson & Moore, 1989). In C4 species, PEPC is fully engaged and CO2 accumulates in the BS at concentrations that are 10– to 20–fold greater than ambient, thereby saturating a fully compartmentalised Rubisco in the BS (von Caemmerer & Furbank, 2003). The biochemical functions of the M and BS need to be separated by a suitable distance (Jurić et al., 2017). Across this space large fluxes of metabolites need to be exchanged, both through plasmodesmata (Osmond & Smith, 1976; Danila et al., 2018), and through a suite of chloroplast membrane transporters (Weber & von Caemmerer, 2010; Gowik et al., 2011; Schlüter et al., 2016).

Quantifying the potential gains from operating a CCM has challenged physiologists for the last 50 years. Simple approaches have compared C3 and C4 plants, but the evolutionary traits of unrelated species can differ substantially, preventing the isolation of the effects of CCMs [reviewed in Snaydon (1991) and Christin and Osborne (2014)]. For instance, in a large comparative experiment Atkinson et al. (2016) found C3 and C4 grasses mainly differed in terms of leaf mass per area, rather than net assimilation rate per unit leaf area, but Taylor et al. (2010) reported that a more limited set of C4 grasses had a 45 % higher assimilation rate than C3 grasses. The comparison is further complicated by the co–occurrence of acclimatory traits: Schmitt and Edwards (1981) reported that the effect of short and long term temperature acclimation was greater than any difference in assimilation
rate between maize and rice. Even in targeted comparisons between rice and the sympatric weed *Echinochloa glabrescens* or crops such as maize, results were inconclusive (Sheehy, 2007; Covshoff *et al.*, 2016). To quantify the benefit of operating a CCM it is therefore critical to compare two plants in which all traits, other than the strength of the CCM, are equal.

For this hypothetical analysis, mathematical models are in principle the ideal tool. Heckmann *et al.* (2013) found a smooth monotonic increase in assimilation for increasing levels of C₄ expression in a C₃ background. This finding was directly dependent on the assumption of unlimited ATP, and contrasts with the observation that C₄ plants are favoured only under high temperatures and light intensities (Monteith, 1978; Pearcy & Ehleringer, 1984). Wu *et al.* (2017) compared predictions of C₃ and C₄ models, but these were parameterised separately by curve fitting on representative C₃ and C₄ crops, thereby replicating the unwanted coexistence of multiple traits present in nature within the models. The light–limited model developed by von Caemmerer (2000) assumed a fixed stoichiometric conversion between electron transport and ATP production and is unsuitable for testing different levels of C₄ engagement because the C₄ cycle requires an increased ratio of ATP to NADPH, which C₄ plants obtain by upregulating cyclic electron flow, CEF (Ishikawa *et al.*, 2016). Recently Yin and Struik (2017) overcame some of these shortcomings, but biochemical processes were relatively schematic, and as a result, metabolite exchange requirements have not been quantified.

The aims of this work were three–fold. Firstly, to develop the theoretical underpinnings of the introduction of CCMs into C₃ crops at the leaf level; secondly, quantify the possible benefits and trade–offs of CCMs if they were to be made operational in rice; and, finally, estimate realistic fluxes to help define targets for expression of enzymes and transporters. Light–limited formulations working under the assumption of limiting ATP or NADPH, as well as enzyme–limited formulations, all valid for any photosynthetic type, are developed here. These are integrated with a mechanistic description of photosynthetic light reactions, and with a biochemical and hydromechanical model of stomatal behaviour. A gas–exchange experiment was used to inform the model. The results predict that introducing CCMs in C₃ metabolism under the current ambient CO₂ concentration would increase assimilation under full light, but the benefit would be reversed at low light intensity (PPFD). For C₄ photosynthesis, achieving this potential will require an appropriate electron transport chain, allowing adequate metabolite traffic, and enlarging the BS to house the biochemical and light harvesting machinery.
Material and Methods

Overview of the modelling approach

The modelling scheme is depicted in Figure 1 to highlight key inputs and outputs. This model was newly derived to allow a seamless transition between all photosynthetic types except CAM, and joins together an electron transport submodel, a biochemical submodel, a stoichiometric submodel (see schematic in Figure S1), and a stomatal submodel. The photosynthetic type is defined by setting the strength of the C4 cycle (as PEP carboxylation rate ($V_{P(J)}$) in the light–limited model and maximum rate of PEPC, ($V_{P,MAX}$) in the enzyme–limited submodel) together with the location of GDC ($\chi_{GDC}$). The electron transport submodel (Note S1) calculates the flux of ATP and NADPH ($J_{ATP}$ and $J_{NADPH}$) made available under a given PPFD. Here, the limitations of previous modelling approaches using a fixed stoichiometry of the electron transport chain (see Introduction) were resolved by allowing the ratio of ATP/NADPH production to be adjusted through mechanisms that were found to be critical in C4 plants. These are the regulation of the rate of cyclic electron flow (CEF) through the parameter $f_{CyC}$, and inducing the NAD(P)H Dehydrogenase–like (NDH) complex (Ivanov et al., 2005; Friso et al., 2010; Munekage et al., 2010) which is characteristic of C4, and not used by C3 plants, operating mainly the PGR5 / PGRL1 pathway (Yamori & Shikanai, 2016) by varying $f_{NDH}$ (the fraction of CEF passing through the NDH complex). The reducing power requirements of nitrogen reduction are implicitly accounted for here as pseudocyclic electron flow (lumped with the water–water cycle, and adjusted through $f_{PseuOCyc}$), in line with Yin and Struik (2012).

The biochemical submodel has different formulations depending on the limitation, sharing common underpinnings (Note S2). There is a formulation for limitation by Rubisco or PEPC carboxylating capacity (commonly referred to as enzyme limitation, Note S3) and two formulations for light–limited photosynthesis, derived under limiting ATP (Note S4) or NADPH (Note S5). Equations for triose phosphate limited photosynthesis (Busch et al., 2018) were omitted for simplicity as they are relevant under low O2 or high CO2 concentrations, or low temperatures (Busch & Sage, 2017), while crops like rice – fertilised and irrigated – generally experience mainly light limitations (Yin & Struik, 2015). Similarly, limitations imposed by the diffusion of metabolites (Retta et al., 2016) were neglected for simplicity, justified by a recent study addressing the introduction of a weak C4 cycle in C3 photosynthesis using a reaction diffusion model that found that any reduction of $A$ due to the effect of diffusion processes was limited (Wang et al., 2017). The ATP and NADH produced during respiration were neglected because they are likely to be consumed by basal metabolism, while NADH imbalances are likely to be dissipated by mitochondrial alternative oxidases (Buckley & Adams, 2011).

Using dummy values (initial values for a converging iteration) for the CO2 concentration at the M carboxylating sites ($C_M$) the light–limited submodel calculates two distinct sets of outputs, under NADPH and ATP limitations. Of those, that resulting in the minimum $V_C$ is taken as output of the
light–limited model. Similarly, starting from $C_M$, the enzyme–limited submodel calculates a full set of outputs using the kinetic characteristics of Rubisco and PEPC as inputs.

Outputs of light–limited and enzyme–limited submodels are joined using a smoothing function to give a continuous output (Note S6), as well as used to calculate $\tau$, a quantity related to the ATP concentration in the M and the BS that acts as the biochemical driver of stomatal response (Note S7). This was included solely to realistically simulate stomatal conductance in a C3 to C4 continuum, but we make no claim about whether $\tau$ offers a faithful mechanistic description of stomatal behaviour. Hydro–mechanical forcing links guard cell responses to the water status and turgor of the leaf, which relate to soil water status and plant hydraulic conductance. The influence of biochemical factors relative to hydro–mechanical forcing is determined by the parameter $\beta$, while stomatal morphology is described by $\chi_S$. The output of the stomatal submodel is stomatal conductance, $(g_S)$ that, together with mesophyll conductance $g_M$, is used to calculate $C_M$, which is iterated. Temperature dependence is simulated with empirical functions (Note S8). For each combination of inputs, the locality of Rubisco between BS and M ($\chi_{Rubisco}$) together with the rate of flow through CEF ($f_{Cyc}$) were fitted to maximise $A$. This resulted in light reactions generating exactly the ATP and NADPH which was consumed by dark reactions, while the ATP–limited model and the NADPH–limited models converged to output the same level of $A$. The outputs of these submodels ($V_{OBS}$, $V_{CBS}$, $V_{OM}$, and $V_{CM}$) were inputted to a generalised stoichiometric model of assimilation (Bellasio, 2017), used to calculate reaction rates, and fluxes across the BS and M interface (Figure S1). Here, three additional inputs partition key processes between the BS and M: $f_{PR}$, for phosphoglycerate reduction; $f_{CS}$, for carbohydrate synthesis; $f_{PPDK}$, for pyruvate phosphate dikinase (Table 1). Model parameterisation and sensitivity are described in Notes S9 and S10, respectively.

Plants, gas exchange, and fluorometry

Plants of *Oryza sativa* subsp. *indica*, modern, high–yielding variety Takanari (Taylaran et al., 2009) were germinated and grown in 1.5 L pots filled with Martins potting mix (80% composted bark, 10% coir, 10% sand, complete fertiliser), in acrylate greenhouses located in Canberra (35°S, 149°E) under natural illumination in April – May 2018. Pots were partially submerged for a third of the depth in polypropylene tubs and watered weekly for six weeks. Gas exchange and fluorescence were measured on a fully expanded leaf with a setup similar to Bellasio and Griffiths (2014b). Briefly, a portable gas exchange system (LI6400XT, Li–Cor, Lincoln, USA) was modified to operate at low CO2 concentrations (see licor.com) and fitted with a 6400–06 PAM2000 adapter, holding a fibre probe in the upper leaf cuvette distant enough to avoid shading. Light was provided by a bespoke red–blue light source, positioned to illuminate uniformly the leaf. Light intensity was measured through an in–chamber Gallium arsenide photodiode, calibrated using a Li–250 light sensor (Li–Cor). Neoprene gaskets were used on both sides of the cuvette. A mixture of 2 % O2 was prepared by mixing ambient air and N2 with a bespoke gas mixing unit (kindly assembled by Suan
Chin Wong). This mix or ambient air was CO2–scrubbed with soda lime and humidified to a dew point of 15–17 °C upstream of the inlet to maintain water vapour pressure deficit around 1 kPa. CO2 was added from a cylinder (Isi, Vienna, Austria), using the CO2 injection unit of the LI6400XT.

PSII yield was measured with a Dual PAM–F (Heinz Walz GmbH, Effeltrich, Germany). Pulse intensity was adjusted to be between 10,000 and 12,000 µmol m⁻² s⁻¹ thereby exceeding the requirements of between 6,000 and 8,000 µmol m⁻² s⁻¹, depending on CO2 and PPFD levels, to saturate the fluorescence signal. Mass flow leaks (Boesgaard et al., 2013) were monitored with a gas flow meter as detailed in Bellasio et al. (2016b), and sealed with a tiny ridge of atoxic gelatine laid between the gaskets and the leaf. Four photosynthetic response curves were measured at 25 °C on n=4 plants as detailed in Bellasio et al. (2016b). A/Ci curves were measured under a PPFD of 1200 µmol m⁻² s⁻¹, light curves were measured under a Ca of 420 µmol mol⁻¹. Flow rate was 490 µmol s⁻¹; CO2 diffusion through the gaskets was compensated by lengthening the tubing of the LI6400XT reference gas.

Results

Gas exchange

The operational conditions of rice plants were characterised by a comprehensive gas exchange experiment, which combined measurements under ambient and low O2. Primary, diffusion leak–corrected data appear as symbols in Figure 2, PSII yield is shown in Figure S3. Overall, rice displayed typical C₃ responses. Under high PPFD (Figure 2A), A was lower under ambient O₂ (closed symbols) than under low O₂ (open symbols) because of photorespiration. The quantum yield for assimilation (the initial slope of the curves), was higher under low O₂ (0.0397±0.0002 and 0.0512±0.0023 under ambient and low O₂, respectively). Under low Ci (Figure 2B), A was higher under low O₂ than under ambient O₂ because of O₂ competitive inhibition of Rubisco. Assimilation saturated at relatively lower Ci under low O₂ (open symbols) than under ambient O₂. The stomatal conductance (gs) measured in A/PPFD curves (Figure 2C) increased monotonically with PPFD showing a saturating response similar to that of the A/PPFD curve. Under varying external CO₂ concentration (Ca), gs decreased non–linearly with slope depending on the O₂ level. Rice had a slightly higher in vivo Sc/O (Table 1) than that found in vitro (Hermida-Carrera et al., 2016) perhaps for the tight association between mitochondria and chloroplasts that evolved to maximise photosynthetic CO₂ recapture (Sage & Sage, 2009; Hatakeyama & Ueno, 2016). Under a PPFD of 500 µmol m⁻² s⁻¹, rice operated at a relatively low V₀/Vc of circa 0.3 [Figure S4, compare with Bellasio et al. (2014)].

Simulating assimilation and stomatal conductance of native C₃ rice
A/Ci and A/PPFD curves responses for rice were simulated in the same conditions used for gas exchange measurements. The model predicted with accuracy A/PPFD (Figure 2A) and A/Ci curves (Figure 2B) measured under ambient O2, but overestimated A/PPFD and A/Ci curves under low O2 and high Ca. We attribute this to triose phosphate limitation, and to the feedbacks regulating the electron transport chain through the quenching of Y(II) under low O2 (Figure S3) which we have addressed in Bellasio (2018) but not considered in this model, for simplicity. The simulated stomatal behaviour captures very well the shape of the stomatal response, in both A/PPFD and A/Ci curves and at both O2 levels.

Simulating gas exchange of C2, C2+C4 and C4 rice

Here, simulations were intended to capture hypothetical best–case scenario, assuming unlimited phenotypic plasticity whereby Rubisco is optimally distributed and electron transport processes fully accommodate CEF and NDH levels. Conditions and fitting routines were the same as used for the C3 simulations. The C2 shuttle and progressive levels of C4 activity were introduced in native rice by manipulating the activity of PEPC (through the inputs $V_{P\text{MAX}}$ and $V_{P(J)}$), the locality of GDC ($\xi_{GDC}$), the engagement of the NDH pathway of electron transport ($f_{NDH}$) and the BS apportioning of light respiration ($f_{RLIGHT}$, see Table 1 for full details). The levels of the fitted inputs $\chi_{Rubisco}$ and $f_{Cyc}$ are shown in Figure S5. These are relevant for bioengineering as they indicate the required physical distribution of Rubisco, and the necessary adjustments to the electron transport chain. A/PPFD curves (Figure 3A) simulated at a Ca of 400 µmol mol–1 intersect around a PPFD of 300 µmol m–2 s–1. Under lower PPFDs C2 A was the highest and C4 was the lowest. Under higher PPFDs A increased proportionally with the level of CCM engagement and was ~22% higher for C4 than C3 at a PPFD of 1500 µmol m–2 s–1. The analysis of A/Ci curves (Figure 3B) revealed expected differences in predicted gas change characteristics between photosynthetic types, with A at Ca lower than ~550 µmol mol–1 being progressively higher for plants operating CCMs at increasing engagement. But the operation of a CCM necessarily sacrifices A under higher Ca. There were striking differences in stomatal conductance, which was around 40% less in C4 than in C3 under a PPFD of 1500 µmol m–2 s–1 and a Ca of 400 µmol mol–1 (Figure 3C), indicating that the same level of A was achieved with lower transpiration and higher water use efficiency, in line with differences between extant C3 and C4 species (Bellasio et al., 2018; Quirk et al., 2018) although in the field there is some negative feedback on the effect on WUE because of temperature changes. The same differences were maintained in the simulated A/Ci curves (Figure 3D). Notably these differences in gs resulted solely from biochemical differences between photosynthetic types (sensed by the quantity $\tau$) while all other parameters were maintained at C3 levels. The operation of the CCMs resulted in an increase in the CO2 concentration in the BS (Figure 3E and 3F) and in the consequent reduction of the ratio between Rubisco oxygenation and carboxylation (Figure 3G and 3H). The output fraction of BS Rubisco carboxylation $V_{CBS}/V_C$, which depends both on $C_{BS}$ and on $\chi_{Rubisco}$, is shown in Figure 3I and 3J. $V_{CBS}/V_C$ was relatively invariant
with PPFD in all photosynthetic types except C4, where it slightly decreased below 500 μmol m\(^{-2}\) s\(^{-1}\) (Figure 3I). In A/Ci curves \(V_{CBS}/V_{C}\) increased at low \(C_{a}\) for C2 and C2+C4 types and decreased at high \(C_{a}\) for the C4 type. Leakiness (the rate of CO2 retrodiffusion from the BS relative to PEP carboxylation rate), of relevance for isotopic studies, (Cernusak et al., 2013; Bellasio & Griffiths, 2014b) is plotted in Figure S6. To isolate any effect of CO2 diffusion through the mesophyll and stomata, these simulations were repeated using \(C_{M}\) as input, and are shown in Figure S7.

**Assimilatory gain/loss of C2, C2+C4 and C4 rice at different temperatures, \(C_{a}\), and PPFD**

This set of simulations explored gains and losses of operating different types of photosynthesis, as compared to C3. Three scenarios were simulated: one of unlimited plasticity of the electron transport chain and two in which some elements of the electron transport chain remain in a C3 configuration. In the best case scenario electron transport processes fully accommodate the ATP demand of different types of CCM through the optimisation of the levels of CEF (\(f_{Cyc}\)) and by allowing expression of the NDH complex in C2+C4 and C4 types (\(f_{NDH}>0\)). Figure 4 shows that operating C2 was beneficial at all temperatures and PPFDs, but gains were generally lower than 10% (Figure 4B), as compared to C3 (Figure 4A). Operating C2+C4 was slightly counterproductive below a PPFD of 450 μmol m\(^{-2}\) s\(^{-1}\) and a temperature of 40°C but allowed substantial gains above (Figure 4C). The range in which operating C4 photosynthesis did not confer net benefits was cutting diagonally below a temperature of 40°C and a PPFD of 500 μmol m\(^{-2}\) s\(^{-1}\) (Figure 4D). The possible gains and losses were much more pronounced for C4 than for C2 and C2+C4 types. In the operation of the C4 cycle most of the energy saved by suppressing photorespiration is consumed by the regeneration of PEP; the resulting balance depends on their relative flux, and can be quantified through the quantum efficiency of assimilation \(Y(CO_2)\), shown on incident light basis in Figure S8. \(Y(CO_2)\) was very similar for C3 and C2 types. C2+C4 and C4 had higher \(Y(CO_2)\) than C3 at high PPFDs, but lower at low PPFDs. Overall, \(Y(CO_2)\) was slightly lower than our previous measurements in tobacco and maize (Bellasio et al., 2016b; Bellasio et al., 2016a), which we attribute to slightly lower \(Y(II)_{LL}\) and \(s\) (Table 1).

We then compared CCM types to C3 assimilation in the temperature and \(C_{a}\) space, under a moderate PPFD of 700 μmol m\(^{-2}\) s\(^{-1}\), meant to capture illumination of an ordinary erect leaf of a modern cultivar in the upper level of the canopy, in the same optimistic scenario of variable CEF and engaged NDH (Figure 4E). C2 assimilation was beneficial at all temperatures and \(C_{a}\) (Figure 4F). Gains were greater than 10% in a relatively broad set of conditions including under ambient \(C_{a}\) at high temperatures. The C4 and C2+C4 types were disadvantageous above a \(C_{a}\) of around 450 μmol mol\(^{-1}\) and below 40 °C – a broader range than under higher PPFD (Figure 3B). The C4 and C2+C4 types were progressively more advantageous at higher temperature and low \(C_{a}\).

Similar simulations were carried out to represent a less optimistic scenario whereby the activity of the NDH complex remained at C3 levels (\(f_{NDH}=0\)) for all photosynthetic types (Figure 5, top
row). The marginal gains were maintained for the C2 type (Figure 5A); however, C2+C4 and C4 types were counterproductive in a broader range of PPFDs roughly cutting below a PPFD of 700 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for the C2+C4 type and 900 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for the C4 type (Figure 5B and 5C).

In a pessimistic scenario, in addition to the incapacity to express sufficient NDH complex \((f_{NDH}=0)\), CCM types were unable to modify the flux through CEF, which remained capped at C3 levels (Figure 5, bottom row). Here, the marginal gains were maintained for C2 photosynthesis (Figure 5D); however, the C2+C4 type was counterproductive below a PPFD of 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), while the C4 type was counterproductive at all PPFDs below a temperature of 30°C (Figure 5E and 5F). Severe losses in excess of 40% were predicted for the C4 type at ordinary temperatures and moderate to low PPFDs.

**Metabolite transport**

Two further sets of simulations estimated the metabolite fluxes between the M and the BS by manipulating the level of C4 engagement through increasing levels of \( V_P \) (Figure 6) so as to represent the full C2+C4 continuum from C2 (left of each panel) to C4 (right of each panel). In a first scenario (Figure 6A), the level of ATP demand in the BS was minimised. In these conditions, phosphoglycerate is not reduced in the BS but diffuses to the M and is reduced therein to dihydroxyacetone phosphate, DHAP. A minimal part of DHAP is used by carbohydrate synthesis, but the majority diffuses back to BS to replenish the sugar phosphates pool. This drives the metabolite exchange between the M and the BS to a maximum. In addition, because phosphoglycerate reduction is the main NADPH sink in the BS, when ATP demand in the BS is minimal, the NADPH demand in the BS is also minimal. This requires by-passing the malate dehydrogenase in the M, and, to maintain the efficiency of the CCM despite the inability to operate the malate shuttle, the CCM works through alanine and aspartate (Bellasio, 2017). This condition is suboptimal because it requires high concentration gradients of aspartate and alanine when malate and pyruvate do not transport CO2 (Arrivault et al., 2017). At low levels of C4 engagement, when \( V_P \) was low, glycine and serine were operating the C2 shuttle. The model predicts that the reducing power generated in the BS by the decarboxylation of glycine, which could not be used by phosphoglycerate reduction because of the insufficient ATP availability, was returned to the M by the malate and pyruvate shuttle in a ‘backward’ C4 cycle. As \( V_P \) increased, the flux of glycine and strength of the C2 cycle [which scales with \( V_P \), see details in Bellasio (2017)] was progressively reduced, diminishing the excess NADPH in BS together with the malate and pyruvate fluxes that decrease to zero with \( V_P \). With the increase in \( V_P \), the fitted fraction of Rubisco carboxylation in BS increased linearly, causing the ratio of ATP demand in BS relative to M to increase linearly (Figure 6C).

An opposite scenario, where fluxes were minimal, was simulated by fitting \( f_{PR} \) and \( f_{CS} \) to minimise the sum of squared flow rates between BS and M (Figure 6B). In these conditions the increase of phosphoglycerate reduction in the BS drove the ATP demand in the BS to a maximum (Figure
The total fluxes were less than half those of the previous case (54 versus 130 μmol m⁻² s⁻¹); the main metabolites to be transported in these conditions were malate and pyruvate, which were the sole compounds to support the CCM while the flux of aminoacids was minimal. Despite the malate and pyruvate shuttle working in full, and exporting reducing power from the M to the BS, the NADPH demand in the BS was high (Figure 6D), requiring substantial linear electron flow in the BS (~18 μmol m⁻² s⁻¹ of NADPH).

Discussion

This work set out to study the theoretical underpinnings of the introduction of CCMs into C₃ metabolism. A model of enzyme and light–limited assimilation was newly derived to account for the stoichiometry of Bellasio (2017), augmented to include the explicit mechanistic description of the electron transport chain (Bellasio, 2018), and a hydromechanical and biochemical model of stomatal conductance recently shown to work for C₃ and C₄ plants (Bellasio et al., 2017). We shall stress four points distinguishing the importance of this work. Firstly, by including a hydromechanical submodel we provide a means to connect plant assimilatory biochemistry to plant hydraulics, allowing the concurrent investigation of photosynthesis and water use. Secondly, this is the only study comparing C₂ performance with C₃, C₂+C₄ and C₄ seamlessly within a single model, offering a further improvement over approaches targeted to specific types. Thirdly, this is the only study estimating the metabolite fluxes necessary to operate the different photosynthetic types. Lastly, the model marries biochemically comprehensiveness (it includes all main reactions of the photosynthetic metabolism) with computational speed, required by larger scale modelling. This model is generally applicable, and will be valuable for ecophysiological and evolutionary studies, but we will address evolution at a later stage. Here, we applied the modelling framework to predict assimilation and metabolite fluxes in a three dimensional environmental landscape (t × Cₐ × PPFD) using parameters derived for rice. Next, we make some general considerations on the introduction of a CCM in C₃ metabolism, and we elaborate on the special case of rice.

There is a pervasive belief that the introduction of C₄ photosynthesis into C₃ plants will unconditionally increase assimilation, supported by models based on the assumption that ATP and NADPH are unlimited (Heckmann et al., 2013). However, decades of comparison between C₄ and C₃ plants have shown that C₃ plants may be advantaged in a range of conditions [e.g. (Ehleringer et al., 1997; Ghannoum et al., 2000; Christin & Osborne, 2014)]. We showed that, when energy budgets were accounted for, C₄ photosynthesis becomes unfavourable at high CO₂ concentrations, low PPFD and low temperatures, and therefore provide a novel theoretical framework to explain such experimental observations.

*Bundle sheath permeability mediates trade–offs imposed by light intensity*
Modern crops like rice have typically a LAI (leaf area per ground area) of 5–6, meaning that the majority of leaves are shaded and, importantly, the overall performance of C4 types will compromise full–light advantages and shade disadvantages. The key parameter governing photosynthetic losses under low PPFD in C4 photosynthesis is BS conductance, $g_{BS}$ (Bellasio & Griffiths, 2014b). $g_{BS}$ controls the flux of CO$_2$ released in the BS that retrodiffuses to the M, called leakage (Farqhuar, 1983). $g_{BS}$ can vary several orders of magnitude in nature and can affect $A$ substantially (Kromdijk et al., 2014; Yin & Struik, 2017), in particular at high levels of CCM engagement (Figure S9). Under high temperature, $g_{BS}$ is reported to increase (Yin et al., 2016), while under low PPFD $V_{p}$ decreases, driven by a reduced rate of ATP production (Bellasio & Griffiths, 2014b). In these conditions, leakage reduces $C_{BS}$, and, in C4 plants, it dissipates energy through the ATP–dependent regeneration of phosphoenolpyruvate required to re–fix the leaked CO$_2$, making the CCM counterproductive (Tazoe et al., 2008; Ubierna et al., 2011; Ubierna et al., 2013; Bellasio & Griffiths, 2014b; Sun et al., 2014; Pignon et al., 2017). In nature, plants minimise the ratio between leakage and metabolite fluxes by preferentially localising plasmodesmata at the interface between M and BS, while apoplastic diffusion is often reduced by the deposition of a gas–tight suberized cell wall (Sowinski et al., 2008; Sowiński, 2013; Danila et al., 2016; Danila et al., 2018). If low $g_{BS}$ may therefore appear desirable (though perhaps difficult to achieve), high symplastic permeability is required to sustain metabolite diffusion [Figure 6, (Weber & von Caemmerer, 2010)], and this dilemma constitutes an efficiency trade–off that is inherent to the C4 CCM – and unavoidable (Bellasio & Griffiths, 2014a). Indeed, to attune leakage to PPFD levels, $g_{BS}$ in maize was found to adjust during growth (Bellasio & Griffiths, 2014b) as well as in adult leaves (Bellasio & Griffiths, 2014a).

**Future CO$_2$ levels**

Rising anthropogenic atmospheric CO$_2$ concentrations will favour C3 assimilation over C4. Apart from the difficulties in predicting future CO$_2$ levels – not addressed here – predicting assimilation under changing CO$_2$ is very difficult. When plants are exposed to a high CO$_2$ level for a long time they may downregulate the pool of Rubisco and PEPC (Ghannoum et al., 2000; Leakey et al., 2004; Long et al., 2006; Leakey et al., 2012), at the same time, producing fewer stomata (Way et al., 2011; Franks et al., 2012)(Quirk, Bellasio and Beerling, Annals of Botany, in press.). There is a growing body of data gained under controlled conditions [e.g. (Bellasio et al., 2018; Quirk et al., 2018)] and in free air experiments [e.g. (Bishop et al., 2015)], yet, responses are species specific and, currently, evidence is not sufficient to generalise acclimation responses of C4 and C3 plants. As a result, it is common practice in climate modelling to take assimilatory responses measured under transient changes in CO$_2$ levels ($A$/C$_a$ curves) as predictive of stable responses of plants grown under different CO$_2$ levels, that is, no large scale models include representation of the physiological acclimation to future CO$_2$ level (Rogers et al., 2017). With this principle, using simple interpolation of the best case scenario shown in Figure 4H, at 25 °C, C4 assimilation would equal C3 assimilation.
at a $C_a$ of 465 µmol mol$^{-1}$, a level that would be exceeded in 2036 according to the A2 scenario of carbon emission mitigation (http://www.ipcc-data.org/observ/ddc_co2.html).

**Strategies for engineering a CCM**

In the face of global warming, the introduction of CCMs in a C3 crop such as rice was proposed as a possible strategy to increase yield (Leegood, 2013; Long *et al*., 2015). An operational C$_2$ shuttle was considered as a first step in bio–engineering, with the final goal of obtaining a fully expressed C$_4$ type. Of the three biochemical C$_4$ subtypes (NADP–ME, NAD–ME, PEPCK), the NADP–ME was chosen as the initial target (Kajala *et al*., 2011), as it is operated by the crops with greatest productivity (Furbank, 2011) and would require introducing a smaller number of enzymes [in M cells carbonic anhydrase, PEPC, malate dehydrogenase, and pyruvate–phosphate dikinase; in BS cells NADP–ME, plus eight transmembrane transporters (Kajala *et al*., 2011)]. Other subtypes require additional enzymes [aspartate and alanine aminotransferase, PEPK, NAD–ME (Wang *et al*., 2014), plus up to three transporters (Schlüter *et al*.)] and were not considered here, but see Bellasio (2017). Traditionally, strategies for engineering a CCM have emphasized the manipulation of dark reactions and the associated genetics (Kajala *et al*., 2011; Leegood, 2013). Here we point to two overlooked factors required for the operation of a CCM, namely anatomy and light reactions.

Firstly, leaf anatomy needs to be adjusted depending on the level of C$_4$ cycle expression. Anatomy and biochemistry of the BS are mutually interdependent (Bellasio & Griffiths, 2014c). The requirement in light harvesting optical cross section depends on the ATP demand, and determines the required BS volume, mediated by the size of the ATP–generating light harvesting machinery, plus the volume of the dark reactions machinery (Bellasio & Lundgren, 2016). Minimal ATP demand in the BS may be desirable as it would require the smallest BS, and therefore require minimum modification of the current rice anatomy, but would lead to the unwanted necessity of high gradients and flux rates, and require the expression of high levels of metabolite transporters (Pick *et al*., 2011). Aiming at a high ATP demand would have the benefit of requiring the minimum expression of transporters but would require the largest electron transport chain, and therefore a more radical modification of the native C$_3$ anatomy. Identifying a desired anatomical target requires therefore first to identify a biochemical ideotype. Each of the two extreme solutions shown in Figure 6 would entail limited operational robustness (Pick *et al*., 2011), as there would not be any freedom to accommodate transient environmental change (Bellasio & Griffiths, 2014c). A ‘robust flexibility’ would be positioned half–way between these two opposite scenarios, for instance where the ATP demand in the BS relative to M is 0.7. The potential ratio of ATP production in the BS relative to M must exceed 0.7 by a considerable safety margin (Bellasio & Lundgren, 2016) to counter changing light conditions (Bellasio & Griffiths, 2014c). To achieve this, the light absorbed in the BS relative to M under white light, must be close to 0.7. Currently, the size and pigmentation of rice BS is insufficient (Bellasio & Lundgren, 2016). A suitable situation was found in maize, which had a BS
pigmentation circa twice that of the M, and allocated ~30% of the total leaf section area to the BS (Bellasio & Lundgren, 2016) and should be considered as the target for C4 rice. Further, reaching the required levels for gBS will require engineering the appropriate density of plasmodesmata (Danila et al., 2016), reducing leakage, and possibly allow for acclimation of gBS during growth (see above). Alternatively, higher efficiency could be reached by operating the C4 cycle only in those parts of the canopy where the PPFD is higher than a given threshold, but this seems difficult to achieve also because it is adopted neither in mature nor in developing maize leaves (Wang et al., 2013).

Secondly, the operation of a C4 cycle will require important modifications to the electron transport chains. We showed that when cyclic electron flow, CEF (fCyc) and the NDH pathway (fNDH) were allowed to vary (Figure 4), the performance of C2+C4 and C4 types was maximal. This optimal scenario reflects the idea that electron transport processes may spontaneously adjust in response to the expression of a CCM, responding to an increase in ATP demand, through flexibility mechanisms inherent in native chloroplasts (Takeuchi et al., 2000). Higher levels of fNDH would benefit C4 assimilation, but may be physiologically implausible, for example because NDH is very expensive to produce and maintain. It is possible, however, that rice does not have the potential to express adequate level of CEF and NDH components. If fNDH is capped at C3 levels the performance of C4 rice will be lower (Figure 5 A–C), and if fCyc is capped at C3 levels A would be depressed even further (Figure 5 D–F).

Considering the complexities and trade–offs of implementing a C4 cycle, C2 rice may be a desirable product of bioengineering efforts. Despite the relative operational simplicity, the engagement of a C2 shuttle always increased assimilation rate, relative to C3. The assimilation gain was relatively small under ambient Ca, but increased with temperature at low Ca (Figure 4F). Although in water–rich rice paddies plants can maintain stomata open and extreme photorespiratory conditions might not occur at mid–latitudes (where temperatures are milder and the subsp. japonica is favoured), they may occur at low–latitudes (where temperatures are higher and the subsp. indica is favoured), and, particularly, for dryland rice, which would probably be the crop to benefit most from the introduction of a C2 CCM. In the simulations, the locality of Rubisco activity, as xRubisco, was adjusted continuously at varying CM always resulting in optimal Rubisco activity. In nature, however, the proportion of Rubisco in the BS may change only on evolutionary timescales and may be plant–specific. Consequently, there may be a trade–off between optimisation for photorespiratory conditions, by compartmentalising more Rubisco to the BS, or for non–photorespiratory conditions by allowing all Rubisco in the M, with easier access to intercellular CO2. Allocating 10% of Rubisco in the BS was a good compromise (Figure S5).

From leaf–level to crop
Upscaling these findings to calculate crop yield will be a challenging task. Firstly, it will require modelling of the canopy light environment (Song et al., 2013), possibly including diel light cycles of fully illuminated leaves (Wu et al., 2017) and the transient illumination in shaded leaves (Pearcy et al., 1997), nitrogen allocation (Buckley et al., 2002; Dewar et al., 2012), the effect of different canopy architectures (Burgess et al., 2017), the response of $A$ and $gs$ to temperature and humidity (Yin & Struik, 2017). Ideally, the description could consider the potential losses due to suboptimal stomatal aperture (Viallet-Chabrand et al., 2016; Bellasio et al., 2017), and the mid–morning depressions of photosynthetic capacity (Horton & Murchie, 2000). The necessity of translating assimilation into grain yield will add further complexities and require a dedicated crop model accounting for root growth, nitrogen uptake, pathogens, as well as the interactions between cultivars and climate (Li et al., 2015; Paleari et al., 2017). There is an urgent need for addressing some of these challenges. This model offers the necessary underpinnings and can be readily used as a submodel for modelling assimilation at higher spatial level.

**Conclusion**

We developed new ATP–limited, and NADPH–limited submodels of assimilation, as well as a light reaction submodel, coupled with a stomatal submodel. The resulting model connects light harvesting to dark assimilatory biochemistry and hydraulics and is valid for any photosynthetic type. The equations were solved analytically and will be valuable for evolutionary as well as ecophysiological studies, and we encourage their use also for larger scale modelling. The model was calibrated and tested on primary gas exchange and fluorescence data measured on rice. By simulating the introduction of CCMs in C₃ metabolism we showed that C₄ photosynthesis becomes disadvantageous under a set of environmental conditions (low light, low temperatures and high CO₂) thus providing theoretical support for decades of ecophysiological observations. For the expression of a CCM to be advantageous, any modifications to dark reactions need to be accompanied by substantial modifications to light reactions. Specifically, engineering an appropriate electron transport chain, with the possibility of expressing the NDH complex and adjusting levels of cyclic electron flow will be required. These will also need to be accompanied by anatomical modifications to accommodate the biochemical and light harvesting machinery and by the expression of suitable levels of transporters to allow adequate metabolite traffic.

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the introduction of the C₄ cycle in rice. CB gratefully acknowledges funding through a H2020 Marie Skłodowska–Curie individual fellowship (DILIPHO, ID: 702755). GDF gratefully acknowledges the ARC Centre of Excellence for Translational Photosynthesis (Grant number CE140100015).

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Author Contributions

CB conceived of the research, performed measurements, developed and coded the models, ran simulations. CB and GDF wrote the paper.

Availability

The model, coded in Excel, is made freely available in Supporting Information. The model does not include ‘live’ scripts and is fully operational in the open access suite ‘Apache Open Office’. 
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Plants, Botany

Evolution: Tripathy in the and 360 Botany Press.

Sheehy, Asada, Leitch, Moore 2013.

Bernacchi 1978.


Table 1. Acronyms, definitions, values, and units used.

<table>
<thead>
<tr>
<th>Symbol / Acronym</th>
<th>Definition</th>
<th>Values / Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Net assimilation</td>
<td>μmol m(^{-2}) s(^{-1}) output</td>
<td></td>
</tr>
<tr>
<td>a(_t)</td>
<td>Total concentration of adenylates in chloroplast</td>
<td>12.7 mmol m(^{-2})</td>
<td>Farquhar and Wong (1984)</td>
</tr>
<tr>
<td>BS</td>
<td>Bundle sheath</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(_{BS})</td>
<td>CO(_2) concentration in the BS</td>
<td>μmol mol(^{-1})</td>
<td>output</td>
</tr>
<tr>
<td>CCM</td>
<td>Carbon concentrating mechanism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEF</td>
<td>Cyclic electron flow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(_{M})</td>
<td>CO(_2) concentration in the M</td>
<td>μmol mol(^{-1})</td>
<td>variable, iteratively found</td>
</tr>
<tr>
<td>D(_{L})</td>
<td>Leaf to boundary layer water mole fraction gradient in the light (10 × VPD in kPa)</td>
<td>10 mmol H(_2)O mol air(^{-1})</td>
<td>gas exchange</td>
</tr>
<tr>
<td>D(_{D})</td>
<td>Leaf to boundary layer water mole fraction gradient in the dark (10 × VPD in kPa)</td>
<td>8.6 mmol H(_2)O mol air(^{-1})</td>
<td>gas exchange</td>
</tr>
<tr>
<td>E(_t)</td>
<td>Total concentration of Rubisco sites</td>
<td>6.7 mmol m(^{-2})</td>
<td>adjusted from Farquhar and Wong (1984) by fitting gas exchange data</td>
</tr>
<tr>
<td>E(_{CBS}/V_C)</td>
<td>Parameter defining the fraction of actual Rubisco carboxylation in BS relative to leaf–level dimensionless</td>
<td></td>
<td>set to equal V(_{CBS}/V_C) output of the enzyme–limited model</td>
</tr>
<tr>
<td>f(_{CBS})</td>
<td>Fraction of C(_{CBS}) going through the NDH complex</td>
<td>dimensionless</td>
<td>fitted to max A</td>
</tr>
<tr>
<td>f(_{BS/C})</td>
<td>Parameter defining the fraction of PPDK activity in the BS relative to leaf–level</td>
<td></td>
<td>assigned</td>
</tr>
<tr>
<td>f(<em>{PR}, f</em>{CS}, f_{Pseudocyc})</td>
<td>Parameter defining the fraction of activity in BS relative to leaf–level, of phosphoglycerate reduction rate, and carbohydrate synthesis</td>
<td></td>
<td>assigned</td>
</tr>
<tr>
<td>f(_{RLIGHT})</td>
<td>Fraction of respiration in the light in BS relative to leaf–level</td>
<td></td>
<td>assigned</td>
</tr>
<tr>
<td>g(_{BS})</td>
<td>Bundle sheath conductance to CO(_2) diffusion</td>
<td>0.00287† mol m(^{-2}) s(^{-1})</td>
<td>Yin et al. (2016)</td>
</tr>
<tr>
<td>GDC</td>
<td>Glycine decarboxylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g(_{M})</td>
<td>Mesophyll conductance to CO(_2) diffusion</td>
<td>0.26† mol m(^{-2}) s(^{-1})</td>
<td>gas exchange</td>
</tr>
<tr>
<td>g(_{h})</td>
<td>Stomatal conductance in the dark</td>
<td>0.047 mol CO(_2) m(^{-2}) s(^{-1})</td>
<td>gas exchange</td>
</tr>
<tr>
<td>h</td>
<td>Stoichiometry of ATP synthase: protons required to synthesize ATP</td>
<td>4.67 protons / ATP</td>
<td></td>
</tr>
<tr>
<td>I(_1), I(<em>2), I(</em>{0})</td>
<td>Light absorbed by PSI and PSII, by PSI, by PSII, by PSI when f(<em>{CBS}=0), by PSII when f(</em>{CBS}=0), μmol m(^{-2}) respectively</td>
<td></td>
<td></td>
</tr>
<tr>
<td>j(<em>{1}), j(</em>{2}), j(_{0})</td>
<td>Electron flow through PSI, and PSII, respectively</td>
<td>μmol m(^{-2}) s(^{-1})</td>
<td>output</td>
</tr>
<tr>
<td>j(_{AP})</td>
<td>Total leaf–level ATP production rate</td>
<td>μmol m(^{-2}) s(^{-1})</td>
<td>output</td>
</tr>
<tr>
<td>j(_{NADPH})</td>
<td>Total leaf–level NADPH production rate</td>
<td>μmol m(^{-2}) s(^{-1})</td>
<td>output</td>
</tr>
<tr>
<td>j(_{SAT})</td>
<td>PPFD saturated electron transport rate</td>
<td>310† μmol m(^{-2}) s(^{-1})</td>
<td>chlorophyll fluorescence (Wei et al., 1994; von Caemmerer, 2000; Galmés et al., 2016; Hermida-Carrera et al., 2016)</td>
</tr>
<tr>
<td>K(_{o})</td>
<td>Rubisco Michaelis–Menten constant for CO(_2) in the liquid phase</td>
<td>8† μM</td>
<td></td>
</tr>
<tr>
<td>K(<em>{O</em>{2}})</td>
<td>Rubisco Michaelis–Menten constant for O(_2) in the liquid phase</td>
<td>335† μM</td>
<td>von Caemmerer (2000)</td>
</tr>
<tr>
<td>K(_{c})</td>
<td>Effective hydraulic conductance from the soil to the epidermis</td>
<td>12 mmol H(_2)O m(^{-2}) s(^{-1}) MPa(^{-1})</td>
<td>Sander (2015)</td>
</tr>
<tr>
<td>K(<em>{CO</em>{2}})</td>
<td>volatility of CO(_2)</td>
<td>30.3† μbarμM(^{-1})</td>
<td></td>
</tr>
<tr>
<td>K(<em>{O</em>{2}})</td>
<td>volatility of O(_2)</td>
<td>833.3† μbarμM(^{-1})</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>Mesophyll</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDH</td>
<td>NAD(P)H Dehydrogenase–like (complex)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(<em>{M}), O(</em>{BS})</td>
<td>O(_2) concentration in M cells or BS cells</td>
<td>210000 or 210000 μmol mol(^{-1})</td>
<td>adjusted from Farquhar and Wong (1984) by fitting gas exchange data</td>
</tr>
<tr>
<td>p</td>
<td>Concentration of photophosphorylation sites</td>
<td>7.5 mmol m(^{-2})</td>
<td></td>
</tr>
</tbody>
</table>
Photosynthetic photon flux density

Respiration in the light, leaf–level, in the BS or in the M, respectively

input parameter defining the activity of PEPCkd relative to \( V_\text{p} \)

Ribulose bisphosphate carboxylase oxygenase

Ribulose–1,5–bisphosphate

lumped energy conversion coefficient (Yin et al., 2009)

Rubisco carboxylation rate, CO₂ saturated, leaf–level, in the BS, in the M, respectively

Rubisco oxygenation rate, total, in the BS or in the M, respectively

Leaf–level actual phosphoenolpyruvate carboxylation rate, inputs to the light–limited model

CO₂ saturated phosphoenolpyruvate carboxylation rate

Potential pool size of RuBP

Yield of PSI extrapolated under zero \( PPFD \)

Yield of PSII extrapolated under zero \( PPFD \)

Yield of PSII modelled empirically by a non–rectangular hyperbola

parameter scaling BS \( O_2 \) evolution to net assimilation attributed to BS activity

Half the reciprocal Rubisco specificity \( \gamma^* = \frac{1}{2 \chi} \)

Curvature of the non–rectangular hyperbola used to model the light–dependence of \( Y(\text{II}) \)

Curvature of the non–rectangular hyperbola used to smooth the combination of light–limited and enzyme–limited models

Specifies the fraction of \( V_{\text{CMAX}} \) which is decarboxylated in the BS.

Fraction of \( I \) shifting from PSII to PSI upon engagement of CEF

parameter defining the fraction of \( V_{\text{CMAX}} \) in BS relative to leaf–level

Parameter lumping turgor to conductance scaling factor and the hydromechanical / biochemical response parameter

Epidermal osmotic pressure

\( \mu \text{mol m}^{-2} \text{s}^{-1} \)

\( \mu \text{mol m}^{-2} \text{s}^{-1} \)

assumed

assumed

assumed

0.38 e/’quanta

2800+MPa/µbar (gas) or 102+mol mol\(^{-1}\) (liq)

\( V_{\text{CMAX}} \) is 93+µmol m\(^{-2}\) s\(^{-1}\)

\( \mu \text{mol m}^{-2} \text{s}^{-1} \)

output

output

output

output

output

gas exchange

gas exchange

gas exchange

chlorophyll fluorescence

output

output

gas exchange

Buckley et al. (2016)

Sage and Khoshravesh (2016)

Buckley et al. (2016)

Bellasio et al. (2017)

† The value shown is at 25 °C but the quantity was made temperature–dependent; ‡ Alternative scenarios in Figure 4
Figures.

**Figure 1.** Modelling framework. Blue boxes show inputs while orange boxes show outputs; grey boxes represent submodels. Inputs with a thick blue outline are made temperature–dependent. Submodels contoured in red are originally developed for this work. Photosynthetic photon flux density ($PPFD$) is an input to the electron transport submodel to calculate the total ATP production rate ($J_{\text{ATP}}$) and the total NADPH production rate ($J_{\text{NADPH}}$). These and dummy values for CO$_2$ concentration at the M carboxylating sites ($C_M$) are fed into the light- and enzyme–limited submodels (Dashed boxes). The outputs from the photosynthesis submodels are used to calculate chloroplastic ATP concentration ($\tau$) and a smoothed combination of the submodels is fed into a stoichiometric submodel to calculate fluxes and reaction rates. $\tau$ is used in the stomatal submodel along with inputs for soil water potential ($\Psi_{\text{Soil}}$) and evaporative demand ($D_S$). The output stomatal conductance ($g_S$) is used to calculate CO$_2$ concentration in the sub–stomatal cavity ($C_i$) from external CO$_2$ concentration ($C_a$) and in turn used to calculate $C_M$, which is iterated. See Table 1 for more abbreviations.
Figure 2. Assimilation and stomatal conductance measured on rice and corresponding simulations for a C₃ photosynthetic type. Panel A: light–response curves. Symbols show the response of $A$ to decreasing PPFD measured under ambient O₂ (closed circles) or 2% O₂ (open circles). Lines show modelled assimilation under ambient O₂ (solid line) or 2% O₂ (dashed line). Panel B: $A/C_i$ curves. Symbols show the measured $A$ at varying levels of CO₂ concentration in the substomatal cavity, $C_i$, under ambient and low O₂. Lines show the corresponding simulations. Panel C: measured and simulated response of stomatal conductance (gs) to PPFD under ambient and low O₂. Panel D: measured and simulated response of stomatal conductance (gs) to external CO₂ concentration, $C_a$, under ambient and low O₂. Symbols show mean ± SE, n=4. For simulated $A/C_i$ curves, $C_a$ was set at 16 levels [between 20 and 1000 μmol mol⁻¹] while PPFD was set at 1200 μmol m⁻² s⁻¹, the same used for gas exchange measurements. For simulated $A/PPFD$ curves, PPFD was set at 18 levels [between 1 and 1500 μmol m⁻² s⁻¹] and $C_a$ was set at 400 μmol mol⁻¹. Temperature was 25°C while $\chi_{Rubisco}$ and $f_{Cyc}$ were fitted for each combination of inputs.
Figure 3. Simulated $A$–response curves. Compared model output for the four photosynthetic types in response to changes in PPFD (Left) or $C_a$ (Right) varied in the same steps of curves above. Four different photosynthetic types were simulated in a best case scenario for bioengineering whereby NDH is expressed ($f_{NDH}>0$), $f_{Cyc}$ and $\chi_{Rubisco}$ are optimal (fitted to max $A$): $C_3$ (black solid line), representing the measured plants; $C_2$ (orange dashed line); $C_2+C_4$ (red solid line); and $C_4$ (blue dash–dot line). Panels A and B: net assimilation. Panels C and D: stomatal conductance. Panels E and F: CO$_2$ concentration in the BS. Panels G and H: Rubisco rate of oxygenation to carboxylation $V_O/V_C$. Panels I and J: fraction of Rubisco carboxylating activity in the BS, relative to total.
Figure 4. Assimilation in the best case scenario. Gains were calculated for 100 combinations of temperature (varied in 10 steps from 16 °C to 43 °C) and PPFD (varied in 10 steps from 1 to 1500 μmol m⁻² s⁻¹), under a $C_a$ of 400 μmol mol⁻¹ (top row), or in 100 combinations of $C_a$ (varied in 10 steps from 150 to 690 μmol mol⁻¹) and temperature (as above), under a PPFD of 700 μmol m⁻² s⁻¹ (bottom row) in a best case scenario whereby electron transport processes fully accommodate for the presence of different types of CCM ($f_{NDH}>0, f_{Cyc}$ is fitted) and Rubisco is optimally allocated (χRubisco is fitted). The gain was expressed as relative to C3 assimilation (Panels A and E), for C2 (Panel B and F) C2+C4 (panel C and G) and C4 (panel D and H).
Figure 5. Assimilation in alternative scenarios. Gains were calculated in the temperature × PPFD space, under a $C_a$ of 400 µmol mol$^{-1}$, expressed as relative to C3 assimilation (Figure 4). Panels A, B, and C show a less optimistic scenario whereby the activity of the NDH complex remain at C3 levels, modelled by setting $f_{NDH}$ at zero for all photosynthetic types. Panels D, E and F show a pessimistic scenario whereby in addition to $f_{NDH}=0$, the fraction of cyclic electron flow ($f_{Cyc}$) was set at C3 levels for all photosynthetic types.
Figure 6. Modelled fluxes between the M and the BS at increasing levels of C4 engagement. In this simulation the C4 CCM was increasingly upregulated by manipulating PEPC activity \( (V_P) \), \( \mu\text{mol m}^{-2}\text{s}^{-1} \) to increase from 0 to 0.2 \( J_{\text{ATP}} \) to represent the C2 to C4 continuum (from left to right of each panel). Panel A simulates a scenario of minimum ATP demand in BS obtained by setting \( r_{\text{PEPCK}}, f_{\text{PR}}, f_{\text{CS}}, \text{and } f_{\text{PPDK}} \) at zero; other inputs represented the operational conditions of PPFD 700 \( \mu\text{mol m}^{-2}\text{s}^{-1} \), 25 °C, and \( C_a=350 \mu\text{mol mol}^{-1} \). Panel B simulates a scenario of minimum sum of squared flow rates between BS and M obtained by fitting \( f_{\text{PR}} \), and \( f_{\text{CS}} \). In these conditions the ATP demand in BS increased substantially, and is shown as relative to the ATP demand in the M in panels C and D. The flux is considered positive when in the M to BS direction for MAL, ASP, DHAP, and GLY, and in the opposite direction for the other metabolites (Figure S1). Note the different scaling of \( y \)-axes.