Original Article

C₄ photosynthesis in C₃ rice: a theoretical analysis of biochemical and anatomical factors

Shuyue Wang¹,², Danny Tholen² & Xin-Guang Zhu¹,³

¹Key Laboratory of Computational Biology, CAS-MPG Partner Institute for Computational Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China; ²Institute of Botany, Department of Integrative Biology, University of Natural Resources and Applied Life Sciences, BOKU Vienna, Gregor-Mendel-Str. 33A-1180 Vienna, Austria and ³University of Chinese Academy of Sciences, Beijing 100049, China

ABSTRACT

Engineering C₄ photosynthesis into rice has been considered a promising strategy to increase photosynthesis and yield. A question that remains to be answered is whether expressing a C₄ metabolic cycle into a C₃ leaf structure and without removing the C₃ background metabolism improves photosynthetic efficiency. To explore this question, we developed a 3D reaction diffusion model of bundle-sheath and connected mesophyll cells in a C₃ rice leaf. Our results show that integrating a C₄ metabolic pathway into rice leaves with a C₃ metabolism and mesophyll structure may lead to an improved photosynthesis under current ambient CO₂ concentration. We analysed a number of physiological factors that influence the CO₂ uptake rate, which include the chloroplast surface area exposed to intercellular air space, bundle-sheath cell wall thickness, bundle-sheath chloroplast envelope permeability, Rubisco concentration and the energy partitioning between C₃ and C₄ cycles. Among these, partitioning of energy between C₃ and C₄ photosynthesis and the partitioning of Rubisco between mesophyll and bundle-sheath cells are decisive factors controlling photosynthetic efficiency in an engineered C₄-C₃ leaf. The implications of the results for the sequence of C₄ evolution are also discussed.

Key-words: 3D anatomy; reaction diffusion process; systems modeling.

INTRODUCTION

In C₃ species, photosynthesis mainly occurs in mesophyll cells of leaves. Carbon dioxide (CO₂) diffuses through stomata and intercellular spaces before it enters these cells and reacts with Ribulose bisphosphate (RuBP) in the chloroplasts to form the 3-carbon compound 3-phosphoglycerate. This reaction is catalysed by RuBP carboxylase–oxygenase (Rubisco) (Lorimer 1981; Hall & Rao 1999). Oxygen can also be fixed by Rubisco and leads to the production of 2-phosphoglycolate. This compound is subsequently recycled to RuBP by the photosynthetic carbon oxidation (PCO) cycle. The latter process is associated with additional energy cost and results in the release of some CO₂, decreasing photosynthetic efficiency by about one third (Ehleringer & Monson 1993).

In C₄ species, the initial fixation reaction is not catalysed by Rubisco, but instead, CO₂ is converted to bicarbonate (HCO₃⁻) in the cytosol of mesophyll cells and subsequently fixed into C₄ acids by phosphoenolpyruvate carboxylase (PEPC). The C₄ acids are transported from mesophyll cell into bundle-sheath cells through plasmodesmata and decarboxylated back to CO₂. This CO₂ is subsequently refixed by Rubisco located in bundle-sheath chloroplasts. The high affinity of PEPC to bicarbonate and the low permeability of the bundle-sheath-to-mesophyll cell interface leads to a high CO₂ concentration in bundle-sheath cells. This high CO₂ concentration allows for much lower Rubisco oxygenation and photorespiration rates (Furbank et al. 2004). Three types of C₄ photosynthesis have been historically distinguished based on the enzymes catalysing the C₄-acid decarboxylation: an NADP-ME type, an NAD-ME type and a PCK type (von Caemmerer & Furbank 2003). In many plants, these different decarboxylation pathways occur simultaneously to varying degrees (Lorimer 1981; Sommer et al. 2012; Muhaidat & McKown 2013; Wang et al. 2014a). In plants relying predominantly on the NADP-ME pathway, such as Sorghum bicolor, Miscanthus and Zea mays (maize), decarboxylation of C₄ acids in bundle-sheath chloroplasts is mainly catalysed by NADP-malic enzyme (NADP-ME). C₄ plants usually have an inherent higher photosynthetic CO₂ uptake rate and higher conversion efficiency of solar energy compared to C₃ plants (Zhu et al. 2008, 2010). Besides higher light use efficiency, C₄ plants have higher use efficiency of water and nitrogen and have higher yields under warmer temperatures (Hibberd et al. 2008). These features suggest that expressing a C₄ pathway in C₃ crop species may be a useful strategy to improve crop yields (Leegood 2002).

In addition to differences in metabolism, C₃ and C₄ plants have different anatomical features. In rice, irregularly arranged, heavily lobed mesophyll cells are located between the bundle-sheath cells. Chloroplasts in these cells occupy about 66% of the protoplasm volume and cover about 97% of cell periphery, which is thought to maximize the diffusive conductance of CO₂ into the stroma (Sage & Sage 2009). Rice bundle-sheath cells contain fewer chloroplasts than mesophyll cells, and the chloroplasts do not form a continuous
boundary around the periphery of the cell, with only 21% to 52% of the cell surface covered by chloroplasts (Sheehy et al. 2008). The leaves of many C₄ plants are characterized by a so-called Kranz anatomy, that is, each vascular bundle is surrounded by an inner ring of large bundle-sheath cells and an outer ring of mesophyll cells (Furbank et al. 2004). C₄ plants have fewer mesophyll cells between neighbouring bundle-sheath cells and the interval distance between neighbouring bundle-sheath cells is shorter (Dengler et al. 1994). The mesophyll cells in, for example, maize are lobed, but not so extensively as in rice (Giannoutsou et al. 2013; Warner et al. 2014). There are fewer chloroplasts in C₄ mesophyll cells compared to those of related C₃ species, and they do not cover the complete cell periphery (Stata et al. 2014).

In addition, maize bundle-sheath cells are larger than in rice and contain large chloroplasts, which are centrifugally (towards the mesophyll cells) arranged (Maai et al. 2011). Generally, low inter-veinal distances and a high ratio between bundle-sheath and mesophyll cell volume are characteristic for C₄ lineages (Griffiths et al. 2013), but it remains unclear whether the rice leaf anatomy precludes an efficient C₄ photosynthetic cycle.

Most of current work related to C₄ engineering focuses on expressing a complete NADP-ME type C₄ metabolism in a C₃ crop (Kajala et al. 2011; Miyao et al. 2011; Sage & Zhu 2011). Biochemical factors that affect this efficiency have been systematically evaluated earlier (Laik & Edwards 2000; Wang et al. 2014b). However, it is not clear whether it is advantageous to engineer such a C₄ metabolic cycle into a typical C₃ leaf without removing the original C₃ metabolic processes and by utilizing a C₃ leaf anatomy. This is an important question to answer because it will determine whether the current C₄ engineering work needs to remove the existing C₃ metabolic cycle from C₃ leaves and whether anatomical changes to the leaf are necessary. von Caemmerer (2003) analysed the efficiency of an engineered single-cell C₄-type concentrating mechanism in rice. They found that a single-cell approach limits the energy efficiency of C₄ photosynthesis, and suggested that compartmentation of CO₂ decarboxylation in the bundle-sheath may be a far more successful strategy. Therefore, in this work we developed a reaction diffusion model that accounts for a two-compartment C₄ metabolism being expressed in a C₃ background, while also accounting for the typical leaf anatomy observed in rice.

### MATERIALS AND METHODS

A number of different approaches were used in this study to describe the rate of photosynthesis in C₃ leaves and in engineered leaves which contain elements of both C₃ and C₄ photosynthesis. Details of the reaction diffusion models, that is, the 3D model structure, the mass balance equations describing the rates of concentration changes of diffusible substrates and also the rate equations, are shown below and in Supplemental Files S1–S3. We also summarized the differences among these models in Table 1.

#### 3D structure

We constructed a two-cell reaction diffusion model of C₃ rice photosynthesis, which consists of a mesophyll cell connected to a bundle-sheath cell. The CO₂ concentration at the mesophyll cell boundaries was assumed to be in equilibrium with the intercellular air space of a typical leaf. Under this assumption, we modelled a typical rice mesophyll cell (Sage & Sage 2009) containing six lobes, and in each lobe we assumed there is a cluster of mitochondria and a layer of chloroplasts that nearly completely covers the cell wall adjacent to intercellular spaces. The lobed mesophyll cell was modelled as a combination of spheres: a central sphere was fused with six peripheral spheres of the same dimensions and at regular distances from each other, representing the cell lobes. The vacuole of the mesophyll cell was located in the middle of the central sphere. Each bundle-sheath cell contained a layer of chloroplasts proximal to the mesophyll cell, followed by two clusters of mitochondria. The vacuole in the bundle-sheath cell was located distal to the mesophyll cell. The 3D geometry of the mesophyll and bundle-sheath cell, including all organelles is shown in Fig. 1.

The radius of each lobe in these cells was 4 μm. The distance from the centre of the central sphere to the centre of each lobe was 5.77 μm, and there was considerable overlap resulting in a total cell volume of 1550.6 μm³. The mesophyll chloroplast surface exposed to intercellular spaces was about 91.7%. The bundle-sheath cell was built as an ellipsoid with length, width and depth being 10 μm, 8 μm and 8 μm, respectively. The surface area of bundle-sheath chloroplast facing the mesophyll cell wall was about 22%. Additional parameters related to organelles in the model are listed in Table S1.

### Table 1. Different model approaches used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Diffusion limitations</th>
<th>Enzyme limited reaction metabolites</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₃ reaction diffusion model</td>
<td>3D reaction diffusion modelᵃ</td>
<td>CO₂, HCO₃⁻</td>
<td>Materials and Methods</td>
</tr>
<tr>
<td>C₃–C₄ reaction diffusion model</td>
<td>3D reaction diffusion modelᵃ</td>
<td>CO₂, HCO₃⁻</td>
<td>Supplemental File S1</td>
</tr>
<tr>
<td>Extended C₃–C₄ reaction</td>
<td>3D reaction diffusion modelᵃ</td>
<td>CO₂, HCO₃⁻, OAA, malate, PEP, pyruvate</td>
<td>Materials and Methods</td>
</tr>
<tr>
<td>diffusion model</td>
<td></td>
<td></td>
<td>Supplemental File S2, S3</td>
</tr>
<tr>
<td>C₃ biochemical model</td>
<td>Resistance modelᵇ</td>
<td>CO₂</td>
<td>Supplemental File S4</td>
</tr>
<tr>
<td>C₃–C₄ biochemical model</td>
<td>Resistance modelᵇ</td>
<td>CO₂</td>
<td>Supplemental File S4</td>
</tr>
</tbody>
</table>

ᵃThe facilitating effect of CA was considered by accounting for the rates of CO₂ hydration and HCO₃⁻ dehydration (Tholen & Zhu 2011).
ᵇThe facilitating effect of CA was considered by assuming full equilibrium between CO₂ and HCO₃⁻ (Evans et al. 2009).
biochemical reactions in the C3 rice model, the respiration rate, and \( v_{c,bs} \neq 0 \) in bundle-sheath chloroplast, \( v_{c,ms} \neq 0 \) in mesophyll chloroplast, \( r_d \neq 0 \) in mesophyll and bundle-sheath mitochondria, and \( v_{o,ms} \neq 0 \) and \( v_{o,bs} \neq 0 \) in bundle-sheath mitochondria and mesophyll mitochondria, respectively.

Similarly, the equation for HCO₃⁻ diffusion and reactions was:

\[
\frac{D_b}{\eta} \nabla^2 \left[ \text{HCO}_3^- \right] = -h
\]

where \( D_b \) (m²s⁻¹) is the liquid-phase diffusion coefficient for bicarbonate, and \([\text{HCO}_3^-]\) (mol m⁻³) is the bicarbonate concentration.

\[ C_3-C_4 \text{ reaction diffusion model} \]

To model C₄ photosynthesis in a C₃ background, the model described above was extended with a rate equation describing the fixation of bicarbonate by PEPC. Strictly speaking, bicarbonate is converted into OAA by PEPC in the mesophyll cytosol. For better comparison with current biochemical models, we followed the assumption that the conversion into HCO₃⁻ is not limiting the C₄ cycle (von Caemmerer 2000). This means that the rate of carboxylation by PEPC (\( v_p \), in mol m⁻³s⁻¹) is accounted for directly in Eqn 1. A more realistic approach will be taken below under ‘Extended C₃–C₄ reaction diffusion model’. Thus, the equation for CO₂ becomes:

\[
\frac{D_c}{\eta} \nabla^2 [\text{CO}_2] = v_{c,bs} + v_{c,ms} + h - r_d - v_p - v_{me} - v_{o,ms} - v_{o,bs}
\]

where \( v_p \) (mol m⁻³s⁻¹) is the rate of carboxylation by PEPC, and \( v_{me} \) (mol m⁻³s⁻¹) is the decarboxylation rate in the bundle-sheath chloroplast. The total rate of decarboxylation in the bundle-sheath chloroplast was constrained to be equal to the rate of carboxylation by PEPC in the mesophyll cytosol. To incorporate the facilitating effect of CA on diffusion in the model (Cowan 1986; Evans et al. 2009; Tholen & Zhu 2011), we described HCO₃⁻ diffusion using Eqn 2. Detailed reaction rate equations are given in Supplemental File S1.

\[ \text{Extended } C_3-C_4 \text{ reaction diffusion model} \]

Current models of C₄ photosynthesis, including the reaction diffusion model described in the previous paragraph, assume that the enzyme-limited rate of C₄ photosynthesis is limited by Rubisco or by PEP carboxylation. We developed an extended C₃–C₄ reaction diffusion model by including the hydration reaction for CO₂ in the C₄ cycle and also adding C₄ related metabolites: oxaloacetic acid (OAA), malate, pyruvate, phosphoenolpyruvate (PEP) and the C₄ related enzymes: PEPC, malate dehydrogenase (NADP-MDH), NADP-ME, pyruvate and phosphate dikinase (PPDK). Biochemical reactions and CO₂ flows are shown in (Fig 2). The equation for CO₂ reactions and diffusion through the liquid phase is then:
The equation for HCO$_3^-$ was:

$$\frac{D_B}{\eta} V^2[\text{HCO}_3^-] = -h + v_{\text{pepc}}$$  \((5)\)

where $v_{\text{pepc}}$ (mol m$^{-3}$ s$^{-1}$) is the rate of the reaction from bicarbonate to OAA catalysed by PEPC. Additional mass balance equations of C$_4$ metabolites are given in Supplementary File S2.

**Rate equations**

**Rate equations for the light reactions**

Electron transport rate (J mol m$^{-2}$ s$^{-1}$) was calculated following Ögren & Evans (1993) and von Caemmerer (2000):

$$J = \frac{I_2 + J_{\text{max}} - \sqrt{(I_2 + J_{\text{max}})^2 - 4\theta I_2 J_{\text{max}}}}{2\theta}$$  \((6)\)

where $I_2$ (mol m$^{-2}$ s$^{-1}$) is the light absorbed by photosystem II (PS II) in the chloroplasts, $J_{\text{max}}$ (mol m$^{-2}$ s$^{-1}$) is the maximum capacity of electron transport chain and $\theta$ is an empirical curvature factor assumed to be around 0.7 (Evans 1989; von Caemmerer 2000). $I_2$ was calculated as:

$$I_2 = I \cdot \alpha \cdot (1 - f)^{-1}$$  \((7)\)

where $I$ (mol m$^{-2}$ s$^{-1}$) is the incident irradiance, $\alpha$ is the absorptance of leaves (assumed to be 0.85) and $f$ is the fraction of absorbed photons that do not drive electron generation and was set at 0.15 (Evans 1987). The factor 1/2 indicates that 50% of the energy is assumed to be absorbed by PS II.

**Rate equations for metabolic processes**

C$_4$ reaction diffusion model. It has been suggested (von Caemmerer 2000; Leegood 2008) that the rice bundle-sheath contributes to photosynthesis. Moreover, in barley, the concentration of Rubisco in bundle-sheath cells is similar to that in mesophyll cells (Koroleva et al., 2000). We therefore assumed that the concentrations and kinetic properties of Rubisco between these two cell types in rice are the same. Rubisco carboxylation rate in mesophyll and bundle-sheath cells were distributed on the basis of their relative chloroplast volume. The maximum carboxylation capacity per unit leaf area by Rubisco was assumed to be 80 $\mu$mol m$^{-2}$ s$^{-1}$. We assumed a linear electron transport chain operates in both mesophyll and bundle-sheath chloroplast and photosynthesis is limited by the amount of ATP available. Assuming a Q cycle operates in the photosystem (Sacksteder et al., 2000; Kramer & Evans 2011), the ratio between proton transport cross thylakoid membrane and electron flow (H$^+$/e$^-$) is 3. The H$^+$/ATP ratio varies (Kramer & Evans 2011), and here we assumed a ratio of 4 (Sheehy et al. 2000). Thus, the e$^-$/ATP ratio for linear electron transport flow is 3/4. We further assumed that the ratio of electron transport rate between bundle-sheath and mesophyll cells equals the ratio between the total volume of mesophyll and bundle-sheath chloroplasts. The CO$_2$ fixation rates in mesophyll ($v_{c,\text{ms}}$ mol m$^{-3}$ s$^{-1}$) and bundle-sheath ($v_{c,\text{bs}}$ mol m$^{-3}$ s$^{-1}$) chloroplasts can therefore be described as:
\[ v_{c,ms} = \min \left( \frac{(J \cdot \frac{3}{4}) f_{j,ms} [CO_2]_{ch,ms} S_{mes} V_{ch,ms} (3 [CO_2]_{ch,ms} + 7 r^\infty)}{S_{mes} V_{ms} (3 [CO_2]_{ch,ms} + 7 r^\infty)}, \frac{V_{c,ms} f_{j,ms} [CO_2]_{ch,ms} S_{mes} V_{ch,ms} [CO_2]_{ms} (7 r^\infty)}{S_{mes} V_{ch,bs} (3 [CO_2]_{ch,bs} + 7 r^\infty)} \right) \]

\[ v_{c,bs} = \min \left( \frac{(J \cdot \frac{3}{4}) f_{j,bs} [CO_2]_{ch,bs} S_{mes} V_{ch,bs} (3 [CO_2]_{ch,bs} + 7 r^\infty)}{S_{mes} V_{ch,bs} (3 [CO_2]_{ch,bs} + 7 r^\infty)}, \frac{V_{c,bs} f_{j,bs} [CO_2]_{ch,bs} S_{mes} V_{ms} [CO_2]_{ms} (7 r^\infty)}{S_{mes} V_{ch,bs} (3 [CO_2]_{ch,bs} + 7 r^\infty)} \right) \]

where \( K_m = K_c (1 + [O_2]/K_o) \), \([CO_2]_{ch,ms}\) (mol m\(^{-3}\)) and \([CO_2]_{ch,bs}\) (mol m\(^{-3}\)) are the CO\(_2\) concentrations in mesophyll and bundle-sheath chloroplasts, \( V_{c,ms} \) (\(\mu\)mol m\(^{-2}\) s\(^{-1}\)) is the maximum carboxylation rate of Rubisco per unit mesophyll surface area exposed to intercellular spaces area, \( V_{c,bs} \) (\(\mu\)mol m\(^{-2}\) s\(^{-1}\)) is the maximum carboxylation rate of Rubisco per unit leaf area, \( f_{j,ms} \) and \( f_{j,bs} \) are the fraction of bundle-sheath chloroplasts and mesophyll chloroplasts volume relative to the total chloroplast volume respectively, \( K_m \) (mol m\(^{-3}\)) is the effective Michaelis–Menten constant for Rubisco in mesophyll cell, \( \Gamma^\infty \) (mol m\(^{-3}\)) is the CO\(_2\) compensation point in the absence of mitochondrial respiration, \( K_c \) (mol m\(^{-3}\)) is the Michaelis–Menten constant for Rubisco carboxylase, \( K_o \) (mol m\(^{-3}\)) is the Michaelis–Menten constant for Rubisco oxygenase, \([O_2]\) (mol m\(^{-3}\)) is the O\(_2\) concentration, \( f_{j,ms} \) and \( f_{j,bs} \) are the fraction of energy partitioned for photosynthetic carbon reduction (PCR) cycle and PCO cycle in mesophyll and bundle-sheath cells respectively, \( S_{mes} \) (m\(^2\) m\(^{-2}\)) is the mesophyll surface exposed to intercellular spaces area per unit leaf area, and \( V_{ch,ms} \) (m\(^3\) m\(^{-2}\)) and \( V_{ch,bs} \) (m\(^3\) m\(^{-2}\)) are mesophyll and bundle-sheath chloroplast volumes per unit mesophyll surface exposed to intercellular spaces area.

The volumetric respiration rate, \( r_d \) (mol m\(^{-3}\) s\(^{-1}\)), was assumed to be equal in mesophyll and bundle-sheath mitochondria, and was described as:

\[ r_d = \frac{R_d}{S_{mes} (V_{ms,ms} + V_{ms,bs})} \]

where \( R_d \) (mol m\(^{-2}\) s\(^{-1}\)) is the respiration rate per unit leaf area, and \( V_{ms,ms} \) (m\(^3\) m\(^{-2}\)) and \( V_{ms,bs} \) (m\(^3\) m\(^{-2}\)) are mesophyll and bundle-sheath mitochondria volumes per unit mesophyll surface exposed to intercellular spaces area.

In the C\(_3\) biochemical model, the rate of photorespiration is calculated as Rubisco carboxylation rate multiplied by the CO\(_2\) compensation point in the absence of respiration, and divided by the chloroplastic CO\(_2\) concentration (von Caemmerer 2000). Assuming that no photorespiratory intermediates are transported between mesophyll and bundle-sheath cells, we represented the local volumetric photorespiration rate \( (v_{ch,ms} \text{ mol m}^{-3} \text{ s}^{-1}) \) in mesophyll mitochondria and \( v_{ch,bs} \text{ mol m}^{-3} \text{ s}^{-1} \) in bundle-sheath mitochondria as the integral of Rubisco carboxylation rate over the chloroplast volume multiplied by \( \Gamma^\infty \) and dividing by the volume of mitochondria (see also Tholen & Zhu (2011)):
<table>
<thead>
<tr>
<th>Name</th>
<th>Symbol</th>
<th>Default value</th>
<th>Units</th>
<th>Notes and references</th>
</tr>
</thead>
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<tr>
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<td>$[O_2]$</td>
<td>0.21</td>
<td>bar</td>
<td>Assuming 21% oxygen</td>
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<td>$D_{b,b}$</td>
<td>$9.52 \times 10^{-10}$</td>
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<td>(Hoofd et al. 1986)</td>
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<tr>
<td>Cell wall thickness of bundle-sheath cells</td>
<td>$d_{w,b}$</td>
<td>$1.5 \times 10^{-7}$</td>
<td>m</td>
<td>Assumed</td>
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<tr>
<td>Diffusion constant of CO$_2$</td>
<td>$D_{c}$</td>
<td>$1.83 \times 10^{-9}$</td>
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<td>(Hoofd et al. 1986)</td>
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<td>Diffusion constant of malate</td>
<td>$D_{mal}$</td>
<td>$1.22 \times 10^{-9}$</td>
<td>m$^{-2}$s$^{-1}$</td>
<td>Assumed</td>
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<tr>
<td>CO$_2$ concentration in intercellular air space</td>
<td>$C_i$</td>
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<td>Air pressure</td>
<td>$P$</td>
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<td>bar</td>
<td>(Tholen &amp; Zhu 2011)</td>
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<tr>
<td>CO$_2$ permeability in chloroplast membranes</td>
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<td>$0.0035$</td>
<td>m s$^{-1}$</td>
<td>(Evans et al. 2009)</td>
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<tr>
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<td>(Tholen &amp; Zhu 2011)</td>
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<td>CO$_2$ permeability in mitochondria membranes</td>
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<td>$0.0035$</td>
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<td>(Evans et al. 2009)</td>
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<tr>
<td>Cytosol pH</td>
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<td>(Tholen &amp; Zhu 2011)</td>
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<tr>
<td>HCO$_3$ permeability chloroplast membranes</td>
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<td>$5 \times 10^{-7}$</td>
<td>m s$^{-1}$</td>
<td>(Felle &amp; Bertl 1986)</td>
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<tr>
<td>HCO$_3$ permeability mitochondria membranes</td>
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<td>$5 \times 10^{-7}$</td>
<td>m s$^{-1}$</td>
<td>(Felle &amp; Bertl 1986)</td>
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<td>Mitochondria pH</td>
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<td>(Tholen &amp; Zhu 2011)</td>
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<td>Effective bundle-sheath cell wall porosity</td>
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<td>Effective mesophyll cell wall porosity</td>
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<td>(Evans et al. 2009)</td>
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<td>Dark respiration</td>
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<td>$4 \times 10^{-7}$</td>
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<td>CO$_2$ solubility</td>
<td>$s_c$</td>
<td>$3.29 \times 10^{-4}$</td>
<td>mol m$^{-3}$Pa$^{-1}$</td>
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</table>
model. Further equations for C4 cycle are described in Supplemental File S2.

**Model parameterization, algorithm for solving the models**

**Model parameterization**

The range of mesophyll surface exposed to intercellular spaces area per unit leaf area ($S_{mes}$) in rice varies between 10 and 24 m$^2$m$^{-2}$ (Hanba et al. 2004; Giuliani et al. 2013). Our model represents a rice leaf with an $S_{mes}$ of 10 (m$^2$m$^{-2}$). Parameters that are related to biochemical and physical processes were taken from Tholen & Zhu (2011). C4 photosynthesis related enzyme kinetic properties were based on Wang et al. (2014b). Plasmodesmata properties and C4 acid diffusion coefficients are assumed in Table 2. For the C3 reaction-diffusion model, we assumed that the partitioning of the ATP between mesophyll and bundle-sheath is linked to the relative volume of chloroplasts in these cells (4.4:1), and the sum of energy fractions allocated to the mesophyll and the bundle-sheath equals 1 ($f_{f,c_ms}$+$f_{f,b_s}$)=1. For the extended C3–C4 reaction-diffusion model, we assumed that the mesophyll chloroplasts had the same amount of energy available as in the C3 case (4.4), but a fraction of this energy has to be allocated to PEP regeneration; we assumed that this fraction was balanced with the demands of the C4 cycle (1:1). This leads to an energy partitioning of 3.4:1:1 for the PCR and PCO cycle in the mesophyll, PEPC regeneration in the mesophyll and the PCR and PCO cycle in the bundle-sheath ($f_{f,c_ms}$+$f_{f,c4_ms}$+$f_{f,b_s}$=1, $f_{f,c_ms}$, $f_{f,c4_ms}$, $f_{f,b_s}$ defaults are given in Table 2).

**Algorithm for solving reaction diffusion models**

Each reaction diffusion model was discretized into 812,424 elements. Biochemical reactions and boundary conditions (Supplemental File S3) were set up for various metabolites and applied to each subdomain. The models were solved using the finite element method by COMSOL Multiphysics, version 4.3b by a time-dependent solver (start at time was zero and end time was 10$^5$s). Solving the model resulted in an estimate of the steady-state concentrations of metabolites at each discrete element of the model structure. Calculation of photosynthetic rate, mesophyll conductance and bundle-sheath conductance are described in Supplemental File S3.

**RESULTS**

**A comparison of reaction diffusion models with classical biochemical models**

We simulated photosynthetic CO$_2$ uptake rate ($A$) at different intercellular CO$_2$ concentrations (A–C$_i$ response curve) for a C3 rice leaf and a C4 rice leaf expressing a C4 metabolic cycle using our C3, C3–C4 and the extended C3–C4 reaction diffusion models, and compared the results with the commonly used biochemical models of photosynthesis (Farquhar et al. 1980; von Caemmerer 2000). The simulation results from the C3 reaction diffusion model were compared with the classical C3 biochemical model. The photosynthetic rate in C3 biochemical model was calculated as the sum of photosynthesis in mesophyll and bundle-sheath cell (Supplemental File S4). The C3 biochemical model predicted slightly higher rates of photosynthesis compared to the C3 reaction diffusion model (Fig. 3a) using the default parameterization for a typical rice leaf (Table 2, Table S1).

We also compared the C3–C4 and the extended C3–C4 reaction diffusion model to a C3–C4 biochemical model. Because a rice leaf expressing a C4 metabolism may still perform some C3 photosynthesis in mesophyll cells, this C3–C4 biochemical model represented an engineered leaf as the...
sum of C₃ photosynthesis in the mesophyll and C₄ photosynthesis in both the mesophyll cell and the bundle-sheath cells (Supplemental File S4). Similar to the C₃ model, the C₃–C₄ reaction diffusion model predicted slightly lower rates of photosynthesis compared to the biochemical model (Fig. 3b). When a more detailed description of C₄ cycle enzymes was included in the extended version of the model, photosynthesis rates increased somewhat (Fig. 3b). A comparison at different light intensities also showed only little differences between the biochemical model and reaction diffusion models (Fig. S1).

The models predict that adding C₄ reactions to the C₃ model increased the predicted rate of photosynthesis when \( C_i \) is lower than about 45 Pa or PPFD is higher than 240 \( \mu \text{mol} \text{m}^{-2} \text{s}^{-1} \) (Fig. 3c, d). Bundle-sheath photosynthesis only contributed little to the total rate of photosynthesis in the C₃ model (Fig. 3e). But the net CO₂ fixation rate in bundle-sheath cells by C₄ photosynthesis contributed significantly to the total assimilation rate (Fig. 3f). The presence of a working CO₂ concentrating mechanism is further supported by the observations that in the extended C₃–C₄ reaction diffusion model, the CO₂ concentration in the bundle-sheath cells was higher than in the mesophyll (Fig. S2b). In addition, the flux of malate into bundle-sheath cells and the net CO₂ fixation rate in bundle-sheath cells reached a maximum at low \( C_i \) (Fig. S2b). The later result contrasts with that for the C₃ reaction diffusion model, where the CO₂ fixation rate in mesophyll and bundle sheath cells increased gradually with increasing \( C_i \) (Fig. S2a). The extended reaction diffusion model also enables predictions of the concentrations

Figure 3. Comparison of reaction diffusion models with commonly used biochemical models. (a) Predicted photosynthetic CO₂ uptake rate (\( A \)) versus intercellular CO₂ partial pressure (\( C_i \)) in the C₃ reaction diffusion model and the classical C₃ biochemical model by Farquhar et al. (1980) (Supplemental file S1) under saturating light. (b) A–C\(_i\) response curves for the C₃–C₄ biochemical model (Supplemental file S1), the C₃–C₄ reaction diffusion model and the extended C₃–C₄ reaction diffusion model under saturating light. (c) A–C\(_i\) response curves for different models under saturating light. (d) Predicted photosynthetic CO₂ uptake rate (\( A \)) versus light intensity (PPFD) in different models. Intercellular CO₂ partial pressure equaled 28 Pa. (e) Predicted net CO₂ fixation rates in the mesophyll cell (MSC) and bundle-sheath cell (BSC) at different intercellular CO₂ partial pressures (\( C_i \)) for the C₃ reaction diffusion model under saturating light. (f) Predicted net CO₂ fixation rates by C₄ photosynthesis and C₃ photosynthesis at different intercellular CO₂ partial pressures (\( C_i \)) for the extended C₃–C₄ reaction diffusion model under saturating light.

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Factors influencing the photosynthetic CO₂ uptake rate of an engineered C₃–C₄ leaf

We further tested how the predicted rates of photosynthesis in the extended C₃–C₄ reaction diffusion model change in response to several anatomical and biochemical factors. With respect to anatomy, we investigated the effect of the amount of mesophyll surface that was covered by chloroplasts, the effect of bundle-sheath cell wall thickness and the effect of permeability of the bundle-sheath chloroplast envelopes. When the amount of mesophyll surface area covered by chloroplasts was modified, total chloroplast and cytosol volumes were kept constant per unit leaf area by increasing the thickness of the chloroplast, and adjusting the vacuole size. In addition, the concentration of all enzymes in these compartments were kept constant with different coverage. We found that decreasing the surface area of the mesophyll covered by chloroplast from 91.7% to 72.8% had a negligible impact on $A$ in the C₃–C₄ reaction diffusion model, even at low $C_i$, although it corresponded to a decrease in mesophyll conductance of nearly 28% (Fig. 4a). A thickening of the bundle-sheath cell wall, or decreased permeability of bundle-sheath chloroplast membrane, had only small effects on $A$, even although the bundle-sheath conductance significantly decreased (Fig. 4b, c, d).

In a C₃ leaf with engineered C₄ metabolism, ATP is used by three processes: the PCR and PCO cycle in the mesophyll ($f_{J,c\text{,ms}}$), the PCR and PCO cycle in the bundle-sheath ($f_{J,b\text{,bs}}$) and for the regeneration of PEP by PPDK in mesophyll chloroplasts ($f_{J,c\text{,4\text{,ms}}}$). The energy partitioning between the existing C₃ metabolic pathway and the introduced C₄ cycle depends on the relative amount of C₃ and C₄ photosynthetic machinery allocated in mesophyll and bundle-sheath cells. In the engineered C₃–C₄ leaf, the default energy partitioning $f_{J,c\text{,ms}} = 63\%$, $f_{J,c\text{,4\text{,ms}}}=18.5\%$ and $f_{J,b\text{,bs}}=18.5\%$ (see details in Materials and Methods). We tested the effect of the energy distribution on photosynthetic rate under the default settings given in Table 2. Maximum photosynthetic rates could be achieved when $f_{J,c\text{,ms}} = 60\%$, $f_{J,c\text{,4\text{,ms}}}=25\%$ and $f_{J,b\text{,bs}}=15\%$ (Fig. 5).

In this study, we assumed that the default Rubisco content in mesophyll and bundle-sheath chloroplasts scales with the volume of the chloroplast ($f_{V,\text{ms}}$, $f_{V,\text{bs}}$ in Table 2), and therefore only 18.5% of the total amount of Rubisco was present in the bundle-sheath cells in the default C₃ leaf. Increasing
the proportion of Rubisco partitioned to the bundle-sheath cells did not increase photosynthetic rates when this energy partitioning was not modified, and even resulted in a decreased photosynthesis when the fraction of Rubisco partitioned to bundle-sheath chloroplasts exceeded about 40% (Fig. 6).

A sensitivity analysis of the energy partitioning was conducted to determine the maximum CO2 assimilation rate under different fractions of Rubisco partitioning to bundle-sheath chloroplasts ($f_{V_{bs}}$) (Fig. 7). As expected, increasing the amount of Rubisco in the bundle-sheath allows for less energy to be allocated to the C3-cycle in the mesophyll and overall higher $A$ (Fig. 7). Interestingly, when more than half of Rubisco was allocated to bundle-sheath chloroplasts, maximum photosynthesis was achieved when no energy was partitioned to C3 photosynthesis in mesophyll chloroplasts. In this case, 60% of the energy was partitioned to PCR and PCO cycle in the bundle-sheath and 40% was partitioned to PEP regeneration by PPDK in mesophyll chloroplasts (Fig. 7).

**DISCUSSION**

**A new modeling framework to explore the consequences of engineering C4 photosynthetic metabolism in a C3 leaf**

This study presents a new modeling framework that couples biochemical reactions with cellular structural features and related gas-diffusion processes in bundle-sheath and mesophyll cells. Compared to the earlier modeling efforts (Laisk & Edwards 2000; Wang et al. 2014b), our new framework explicitly considers the anatomical features, diffusional processes, in addition to the biochemical processes. A recent two-dimensional reaction diffusion model of a maize leaf was built to explore the effect of diffusion and biochemistry in C4 plant (Retta et al. 2016). Here, we presented a full 3D model that not only enables study of the intricate interaction between different biochemical and anatomical features, but also enables evaluation of the impact of modifying these different features on the photosynthetic efficiency.

We compared the results from the reaction diffusion model with the classical biochemical model of photosynthesis (Farquhar et al. 1980; von Caemmerer 2000). When a normal C3 rice metabolism was simulated by the models, there were very few differences in the photosynthetic rate between both

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**Figure 5.** The predicted photosynthetic CO2 uptake rate ($A$) at an intercellular CO2 partial pressure of 28 Pa versus the fractions of energy partitioned to the PCR and PCO cycle in the mesophyll ($f_{J,c_{ms}}$) and the bundle-sheath ($f_{J,bs}$) in the extended C$_3$–C$_4$ reaction diffusion model under saturated light. The interval used during the sensitivity analysis was 0.05 for each parameter ranging from 0 to 1. The sum of $f_{J,c_{ms}}$, $f_{J,bs}$ and the fraction of energy partitioning for PEP regeneration by PPDK ($f_{J,c4_{ms}}$) is 1.

**Figure 6.** The predicted photosynthetic CO2 uptake rate ($A$) versus the fraction of Rubisco partitioned to bundle-sheath chloroplast ($f_{V_{bs}}$) at an intercellular CO2 partial pressure of 28 Pa for the extended C$_3$–C$_4$ reaction diffusion model under saturating light. The dashed vertical line indicates the default value of $f_{V_{bs}}$ given in Table 2.

**Figure 7.** The maximal photosynthetic rate ($A$, red line) achievable with an optimal energy partitioning (determined from an analysis as shown in Fig. 5) for different fractions of Rubisco partitioning to bundle-sheath chloroplasts ($f_{V_{bs}}$) in the extended C$_3$–C$_4$ reaction diffusion model under saturating light. The energy partitioning (PCR and PCO cycle in the mesophyll ($f_{J,c_{ms}}$), PEP regeneration in the mesophyll ($f_{J,c4_{ms}}$) and PCR and PCO cycle in the bundle-sheath ($f_{J,bs}$)) required for this optimal rate is also shown.
models (Fig. 3a). These differences were attributed to the more realistic representation of the 3D leaf structure in a reaction diffusion model, as compared to the biochemical model where the resistances of all components (the cell wall, cytosol, chloroplast envelope, etc.) were considered as serial and simply added (Supplemental File S4). In addition, (photo)respiratory CO₂ release may result in a variable effective mesophyll conductance (Tholen & Zhu 2011; Tholen et al. 2012), which was not accounted for in the classical biochemical model. Similarly, when adding PEP carboxylation and decarboxylation to the models, a difference between the C₃-C₄ reaction diffusion model and the classical biochemical model was observed (Fig. 3b). The explanation for these observations was again the difficulty in accurately estimating and describing the diffusion resistances between the different compartments (i.e. the resistance to CO₂ leakage) using the biochemical model.

To enable a comprehensive evaluation of the potential factors controlling photosynthetic efficiency in a leaf where C₄ photosynthetic metabolism is engineered into a C₃ metabolic background, we developed an extended C₃-C₄ reaction diffusion model where both the 3D anatomical features and the key enzymes involved in the C₄ cycle. Although the influence of engineering any particular C₄ component on a leaf can be studied directly through a transgenic approach, a mechanistic model can be used to quickly study the expected consequences of genetic engineering. Our extended C₃-C₄ reaction diffusion model reached a stable solution and can predict commonly observed A–Cᵢ curve and light response curve (Fig. 3). It is worth noting that the mesophyll conductance in the extended C₃-C₄ reaction diffusion model (Fig. 4) was in the range of recent estimates for mesophyll conductance in C₄ plants (Barbour et al. 2012).

To illustrate the added capacity of this new reaction diffusion model, we first used it to predict the impact of modifying CA on the rate of photosynthesis (Fig. S4). The concentration of cytosolic CA in the model was based on current estimates for C₃ plants (see Tholen & Zhu (2011) for a discussion). In our simulation, a reduction of the CA concentration by 98% resulted in only a small decrease (11% at ambient CO₂ conditions) in photosynthesis (Fig. S4). This is consistent with a recent report showing that decreasing the activity of CA in maize by about 97% does not influence photosynthesis much under current or elevated CO₂ concentrations (Studer et al. 2014). However, it is worth noting as well that photosynthetic rate was drastically decreased when CA is less than 5% of wild type in C₄ dicot plants Flaveria bidentis, which has 10-fold higher CA activity than maize (von Caemmerer et al. 2004; Cousins et al. 2008). This difference in the control of CA over photosynthetic efficiency might be the result of alternative mechanism in C₄ monocots evolution (see the discussion in (Studer et al. 2014)).

Factors influencing the photosynthetic efficiency of an engineered C₃-C₄ leaf

To test whether the above conclusions are robust, we performed a sensitivity analysis for a number of anatomical and biochemical features used in the reaction diffusion model. Firstly, we examined the influence of chloroplast coverage adjacent to intercellular spaces on the conductance to CO₂ diffusion and on the rate of photosynthesis. In C₃ plants, mesophyll conductance is thought to correlate well with the proportion of chloroplast area exposed to intercellular air spaces (Laisk et al. 1970; Terashima et al. 2006). Rice has a high proportion of chloroplast surface area covering cell wall (Sage & Sage 2009), which might have been the result of a strong selection pressure. In C₄ plants, however, high chloroplast coverage may be counter-productive as it would decrease the amount of cytosol (and thus PEPC) adjacent to the point of CO₂ entry. Mesophyll cells of C₄ plants commonly have fewer chloroplasts, and these chloroplasts are located further from the cell walls (Stata et al. 2014), von Caemmerer (2003) attributed the inefficient C₄ photosynthesis in a single C₃ cell partly to the large chloroplast surface coverage. Our results show that the conductance to CO₂ diffusion between the intercellular airspace and site of fixation increases with the amount of chloroplast coverage for C₃ plants (Fig. S5), but indeed decreases with coverage in C₄ photosynthesis in C₃ plants. However, both effects on assimilation rates were only minor (Fig. 4, S5), suggesting that the chloroplast coverage in the mesophyll would not significantly limit pressure in the intercellular airspaces rises above 45 Pa (which is far above the levels corresponding to current atmospheric conditions) (Fig. 3c). Furthermore, under ambient CO₂ levels, that is, C₃ = 28 Pa, photosynthetic rate was enhanced when light intensity is above 240 μmol m⁻² s⁻¹ (Fig. 3d). The higher predicted A was because of the CO₂ concentrating mechanism, as demonstrated by the elevated CO₂ concentrations in the bundle sheath cell and also a lower CO₂ saturating point in the extended C₃-C₄ reaction diffusion model, as compared to the C₃ reaction diffusion model (Fig. S2). Although adding a C₄-cycle into a C₃ leaf decreased the CO₂ assimilation in mesophyll cells, the total photosynthetic rate increased compared to the default C₃ leaf (Fig. 3c, e, f). These results show that expressing a C₄ metabolic cycle in a C₃ leaf can increase photosynthesis, even without removing the original C₃ metabolism or making extensive changes to the mesophyll anatomy. Therefore, although expressing a single-cell C₄-cycle in the mesophyll is unlikely to improve photosynthesis (von Caemmerer 2003), our results show that compartmentation of the C₄ metabolism in a mesophyll and bundle-sheath part in a C₃ leaf allows for higher A. However, it is important to note that here we assumed that every mesophyll cell was connected to a bundle-sheath cell. The rice mesophyll has much larger numbers of mesophyll cells per bundle-sheath (Smillie et al. 2012), and this anatomical feature would reduce the efficiency of such a C₄ rice plant. Thus, increasing vein density (Tolley et al. 2012) is necessary to achieve higher rates of photosynthesis in such plants.

Engineering C₄ metabolism into a C₃ leaf can lead to increased photosynthetic rates

When a two-cell C₄-cycle was added to an existing C₃ metabolism, the model predicted that photosynthesis under saturating light conditions would be enhanced until the CO₂ partial
CO₂ uptake and might not need to be modified during C₄ engineering.

In C₄ plants, the efficiency of the C₄ cycle is limited by leakage of CO₂ or bicarbonate from the bundle-sheath back into the mesophyll (Farquhar 1983; von Caemmerer 2000; Kromdijk et al. 2008; Bellasio & Griffiths 2014). If leakage is high, more energy needs to be spent to maintain a high CO₂ concentration in the bundle-sheath, resulting in less efficient photosynthesis. Given this importance of leakage to C₄ efficiency, many C₄ plants have anatomical features that prevent excessive leakage of carbon from the bundle-sheath, such as suberized lamellae between the bundle-sheath and mesophyll cell walls, or centripetally arranged chloroplasts in the bundle-sheath cells (Leegood 2002). In addition, the diffusion across cell and chloroplast membranes is facilitated by aquaporins acting as a CO₂-pore (Kaldenhoff 2012), and this opens possibilities for differences in membrane permeability or its regulation between C₃ and C₄ plants. Single-cell C₄ plants similarly have anatomical adaptations that spatially separate the initial CO₂ fixation reaction from the decarboxylation reaction near Rubisco (Edwards et al. 2004).

To investigate whether features described above influence the efficiency of a C₃ plant expressing a C₄ cycle, we varied the thickness of the cell wall, and the permeability of the bundle-sheath chloroplast membranes to CO₂ and bicarbonate. The results show that although an increased permeability of the wall or membranes increased the conductance to carbon, the effect on photosynthesis was minor (Fig. 4). Thus, our results indicate that a mixed C₃–C₄ metabolism does not benefit much from a high resistance between bundle-sheath and mesophyll. This is because in the engineered C₃–C₄ leaf, the CO₂ uptake rate contributed by the C₄ photosynthesis only accounts for minor (about one third) of the total photosynthetic rate when C₄ equaled 28 Pa (Fig. 3). Effects of the bundle-sheath cell wall thickness and bundle-sheath chloroplast permeability for bicarbonate on the photosynthesis are more significant when more Rubisco and energy are allocated to the bundle-sheath (Fig. S6).

An efficient C₄ metabolism must optimize the energy allocation between PEP regeneration in the mesophyll and the PCR and PCO cycle in the bundle-sheath. We examined what would be the optimal energy distribution among PEP regeneration, the PCR and PCO cycle in the mesophyll and in the bundle-sheath. Our results indicate that if 18.5% of the Rubisco is partitioned in bundle-sheath cells (default value in Table 2), allocating about 50% of the available energy to the C₄ cycle leads to optimal A (Fig. 7). If the amount of Rubisco in the bundle-sheath can be increased over that in mesophyll cells, correspondingly more energy is required in C₄ photosynthesis, then there is no need to maintain C₃ photosynthesis in the mesophyll (Fig. 7). These results suggest that a coordinated partitioning of Calvin-cycle enzymes and energy is an important aspect for achieving improved rates of photosynthesis with C₄ engineering. One caveat in this study is that the transfer of PGA/triose phosphate between bundle sheath cell and mesophyll cell, as discussed in a recent C₄ metabolism model (Wang et al. 2014b), was not considered. Therefore, the actual energy partitioned into the bundle sheath cell would be higher than the value shown in Fig. 7 because part of the PGA generated in BSC will be phosphorylated and reduced in the mesophyll cells. We also analysed the optimal energy distribution under different light intensities. Our results indicate that under low light, more energy has to be partitioned to C₃ photosynthesis to achieve maximal A (Fig. S7). This is a consequence of C₃ photosynthesis needing less ATP compared to C₄ photosynthesis.

For each CO₂ fixed, C₄ photosynthesis requires an additional 2 ATP to maintain the carbon concentrating mechanism. The extra ATP requirement can be met by an increased capacity of cyclic electron transport in NADP-ME type C₄ plants compared to C₃ plants (kanai & Edwards 1999; nakamura et al. 2013). In our current analysis, we focused on examining the role of diffusion and enzyme limited biochemistry on the photosynthetic rates in an engineered C₃–C₄ leaf. An analysis of the effect of changes in the electron transport stoichiometry is beyond the scope of our work. Models have been developed that describe the required cyclic electron transport in C₃ and C₄ leaves (Zhu et al. 2005; Yin & Struijk 2012; Walker et al. 2014). However, because of the current incomplete understanding of the regulation of electron transport and the interaction between cyclic and linear electron transfer capacity, a fully mechanistic model of this process is yet to be developed.

### Implications for the evolution and engineering of C₄ photosynthesis

C₄ photosynthesis differs from C₃ photosynthesis in many anatomical and biochemical aspects (Sage & Zhu 2011). The current notion is that these features were acquired in a stepwise manner during the C₄ evolutionary processes (Sage et al. 2012). Mallmann et al. (2014) suggest that the re-balancing the nitrogen metabolism between bundle sheath and mesophyll cells after the re-localization of glycine decarboxylase from mesophyll to bundle sheath cells might have been a major evolutionary driving force for the establishment of the C₄ metabolic cycle. The present study suggests that formation of a C₄ metabolic cycle in a C₃ photosynthetic background can also lead to increased photosynthetic efficiency and hence function as an evolutionary driving force (Fig. 3). This benefit is larger under low CO₂ concentrations (Fig. 3), which is consistent with the report that C₄ species emerged during the Oligocene period, a geological period that is feature by a low atmospheric CO₂ concentration (Christin et al. 2008).

The results obtained from this simulation study are consistent with the current understanding of the evolutionary trajectories of C₄ photosynthesis. The metabolic structure of the simulated C₃–C₄ species in this study mimics C₄-like species, where a C₄ photosynthetic metabolism is incorporated into a C₃ background without establishment of cell specific expression of photosynthetic enzymes, in particular Rubisco, between the bundle sheath and mesophyll cells (Sage et al. 2012). After the formation of C₄-like species, photosynthetic efficiency is further optimized through establishment of cell specific expression patterns for key photosynthetic enzymes, e.g. Rubisco (Sage et al. 2012). Our results show that further redistribution
of Rubisco content between bundle sheath and mesophyll cells can lead to increased photosynthesis, and this is consistent with such an evolutionary sequence (Fig. 7). In addition, we found the percentage of energy required for maximal C4 photosynthesis in the C3–C4 rice leaf increased with more Rubisco distributed to bundle-sheath cell. Thus, increased energy partitioning to the bundle-sheath is an expected trend during C4 evolution.

CONCLUSIONS

We developed a 3D reaction-diffusion model by incorporating a NADP-ME-type C3 metabolism and a C3 biochemical model in a realistic geometry representing part of a rice leaf. The model was then used to explore the influence of anatomical and biochemical features of an engineered C4 rice leaf. Our results suggest that expressing a two-cell C4 metabolism in a C3 rice leaf may lead to an increased photosynthetic efficiency. Furthermore, we found that Rubisco allocation and energy partitioning are crucial to gain an increased photosynthesis in the engineered leaf.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Chinese Academy of Sciences and the Max Planck Society for support. Funding for the authors’ research is from the Bill & Melinda Gates Foundation (Grant No. Opp1014417, OPP1129902), EU FP7 3 to 4 project (EU 289582), the CAS strategic leading project “Modular DESIGNer Crop Breeding” (Grant No. # XDA08020301), Ministry of Science and Technology of China (#2015CB150104) and International Max Planck Research Schools.

AUTHOR CONTRIBUTIONS

Xin-Guang Zhu, Danny Tholen and Shuyue Wang designed the research. Shuyue Wang developed the model and performed the analysis. Shuyue Wang, Danny Tholen and Xin-Guang Zhu wrote the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES


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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Table S1.** Additional anatomical and biochemical parameters in the reaction diffusion and biochemical models (at 25 °C).

**Figure S2.** Comparison of the predicted photosynthetic CO₂ uptake rate (A) versus light intensity (PPFD) in the C₃–C₄ biochemical model, C₃–C₄ reaction diffusion model and the extended C₃–C₄ reaction diffusion model. Intercellular CO₂ partial pressure equaled 28 Pa.

**Figure S3.** Metabolite concentrations (OAA: oxaloacetic acid; PEP: phosphoenolpyruvate; malate and pyruvate) in specific organelles versus intercellular CO₂ partial pressure (Ci) predicted by the extended C₃–C₄ reaction diffusion model under saturating light. MS: mesophyll cell; BS: bundle-sheath cell.

**Figure S4.** Predicted photosynthetic CO₂ uptake rate (A) at saturating light versus intercellular CO₂ partial pressure (Ci) in the extended C₃–C₄ reaction diffusion model at default and at low (2% in mesophyll cell) CA levels.

**Figure S5.** Net CO₂ fixation rate (continuous line) and mesophyll conductance between intercellular airspaces and the site of initial CO₂ fixation (dashed line) versus the fraction of the mesophyll surface that is covered by chloroplasts (Sc/Smes) for the C₃ reaction diffusion model under saturated light. Intercellular CO₂ partial pressure was 28 Pa. Five different model geometries (indicated by the points) were analysed. The dashed vertical line indicates the default coverage.

**Figure S6.** The effect of anatomical features, including surface area of chloroplasts exposed to intercellular spaces relative to surface area of mesophyll cells (Sc/Smes), bundle-sheath cell wall thickness (dbw), bundle-sheath chloroplast envelope permeabilities to CO₂ (Pco₂) and bicarbonate (Pbic) on photosynthesis (A) and conductance for the extended C₃–C₄ reaction diffusion model with different fractions of Rubisco partitioned in bundle-sheath chloroplast (fV bs). (a, b) Net photosynthetic CO₂ uptake rate (A) (a) and mesophyll conductance (gm) (b) between intercellular airspaces and the site of initial CO₂ fixation versus the mesophyll chloroplast coverage adjacent to intercellular spaces. (c, d) The predicted fV bs and bundle-sheath conductance (d) versus bundle-sheath cell wall thickness. (e, f) Predicted A (e) and bundle-sheath conductance (f) for different bundle-sheath chloroplast membrane permeabilities to CO₂. (g, h) Predicted A (g) and bundle-sheath conductance (h) for different bundle-sheath chloroplast membrane permeabilities to bicarbonate. Simulations were done at an intercellular CO₂ partial pressures as 28 Pa under saturating light. Corresponding energy partitioning with different fV bs was from Fig. 7. Other parameters were taken from Table 2.

**Figure S7.** The maximal photosynthetic rate (A, red line) achievable with an optimal energy partitioning (determined from an analysis as shown in Fig. 7) for different light levels. The energy partitioning (PCR and PCO cycle in the mesophyll (fJ c₃ ms), PEP regeneration in the mesophyll (fJ c₃ ms) and PCR and PCO cycle in the bundle-sheath (fJ bs)) required for achieving these rates is also shown.

**File S1:** Reaction rates in the C₃–C₄ reaction diffusion model.

**File S2:** Mass balance equations for C₄ acids and rate reactions of the C₄ cycle in the extended C₃–C₄ reaction diffusion model.

**File S3:** Boundary conditions and calculation of conductance for reaction diffusion models.

**File S4:** Biochemical models for C₃ and C₃–C₄ photosynthesis.