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A partial C_4 photosynthetic biochemical pathway in rice

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Abstract

Introduction of a C_4 photosynthetic pathway into C_3 rice (*Oryza sativa*) requires installation of a biochemical pump that concentrates CO_2 at the site of carboxylation in modified bundle sheath cells. To investigate the feasibility of this, we generated a quadruple line that simultaneously expresses four of the core C_4 photosynthetic enzymes from the NADP-malic enzyme subtype, phospho*enol*pyruvate carboxylase (*Zm*PEPC), NADP-malate dehydrogenase (*Zm*NADP-MDH), NADP-malic enzyme (*Zm*NADP-ME) and pyruvate phosphate dikinase (*Zm*PPDK), in a cell-specific manner. This led to enhanced enzyme activity but was largely neutral in its effects on photosynthetic rate and growth. Measurements of the flux of $^{13}CO_2$ through photosynthetic metabolism revealed a significant increase in the incorporation of ^{13}C into malate, consistent with increased fixation of $^{13}CO_2$ via PEP carboxylase in lines expressing the maize PEPC enzyme. We also showed ^{13}C labelling of aspartate indicating additional $^{13}CO_2$ fixation into oxaloacetate by PEPC and conversion to aspartate by the endogenous aspartate aminotransferase activity. However, there were no significant differences in labelling of 3-phosphoglycerate (3PGA) or phospho*enol*pyruvate (PEP) indicating limited carbon flux through C_4 enzymes into the Calvin-Benson cycle. Crossing the quadruple line with a line with reduced glycine decarboxylase H-protein (*Os*GDCH) abundance led to a photosynthetic phenotype characteristic of the reduced *Os*GDCH line and higher labelling of malate, aspartate and citrate. While Kranz anatomy or other anatomical modifications have not yet been installed in these plants to enable a fully functional C_4 cycle, these results demonstrate for the first-time flux through the carboxylation phase of C_4 metabolism in transgenic rice containing the key metabolic steps in the C_4 pathway.

Keywords: C_4 rice, C_4 photosynthesis, ^{13}C labelling, NADP-malic enzyme, malate, *Oryza sativa* (rice), transgenic rice, metabolic engineering.

Introduction

A major recent research objective has been the engineering of a C_4 photosynthetic pathway into rice (https://C4rice.com; Kajala *et al*., 2011; von Caemmerer *et al*. 2012; Ermakova *et al*. 2019), potentially leading to an increase in radiation use efficiency and yield of up to 50% (Hibberd *et al.* 2008). The C_4 pathway represents a complex combination of both biochemical and anatomical adaptations that suppresses photorespiration by effectively saturating ribulose bisphosphate carboxylase/oxygenase (Rubisco) with CO_2. In the majority of C_4 plants, this is achieved by compartmentalization of photosynthetic reactions between two morphologically distinct cell types: the mesophyll cells (MCs) and the bundle sheath cells (BSCs). Operating across these cells is a biochemical CO_2 pump elevating the CO_2 concentration in the BSCs where Rubisco is located (Hatch 1987).

There are three primary variants of this pump characterized by the main decarboxylase reaction (Hatch *et al*. 1975). The NADP-ME subtype was chosen for engineering C_4 photosynthesis into rice as it is well-characterized in the C_4 model crop species maize (*Zea mays*) and potentially requires the fewest biochemical enzymes among all C_4 subtypes (Kajala *et al*., 2011; Weber and von Caemmerer, 2010; Ermakova *et al.* 2019). Each molecule of CO_2 entering the cytosol of the MCs is first converted to bicarbonate (HCO_3^-) by the activity of carbonic anhydrase (CA) and then incorporated into phospho*enol*pyruvate (PEP) by PEP carboxylase (PEPC), yielding the C_4 acid oxaloacetate (OAA). OAA is taken up into the chloroplast of the MCs where it is reduced to malate by the NADP-dependent malate dehydrogenase (NADP-MDH). Malate is exported back to the cytosol and then diffuses into BSCs through plasmodesmata along a steep concentration gradient. In the BSCs, malate is transported into the chloroplast by an unknown transporter and oxidatively decarboxylated by NADP-dependent malic enzyme (NADP-ME), yielding CO_2, NADPH and pyruvate. CO_2 is assimilated by Rubisco, yielding two molecules of 3PGA, about half of which is reduced to triose-phosphate (TPs) using the NADPH provided by NADP-ME in the BSC chloroplast to regenerate RuBP in the Calvin-Benson cycle. The other half of the 3PGA moves to the

MCs for reduction to TP in the MC chloroplast and then returns to the BSCs to enter the Calvin-Benson cycle. Pyruvate moves from the BSCs into the chloroplasts of the MCs where it is converted to PEP by pyruvate:phosphate dikinase (PPDK).

In this study, we report on the introduction of part of this biochemical pathway into rice. It has previously been shown that genomic sequences encoding C_4 proteins give stronger expression in rice than cDNAs (Matsuoka *et al*. 1994). Therefore, we decided to express individual full-length genes of *Zm*PEPC, *Zm*PPDK, *Zm*NADP-MDH and *Zm*NADP-ME (including promoters, untranslated regions, exons and introns) from maize (Kajala *et al*. 2011) in a bid to achieve a C_4-like pattern of C_4 gene expression, enzyme localization, enzyme activity and enzyme kinetic properties (Miyao 2003, 2011). Individual lines were then crossed to generate a plant overexpressing all four of these core C_4 cycle enzymes to investigate the feasibility of installing a functional C_4 biochemical pathway into rice. This quadruple transgenic line was also crossed with a line with decreased *Os*GDCH protein (Lin *et al*. 2016), and this quintuple line was used to test whether lowering GDC in the MCs of rice primes the plant for introduction of a C_4 cycle (Sage 2004; Sage *et al*. 2012). We investigated the effect on plant growth and photosynthesis.

To evaluate photosynthetic functionality, we used $^{13}CO_2$ labelling experiments (Arrivault *et al.* 2017), similar in concept to the radiolabelling experiments originally performed to characterize flux in C_4 photosynthesis (Hatch *et al*. 1967, Hatch 1971). Flux of $^{13}CO_2$ through photosynthetic metabolism, in particular into C_4 acids, was determined for the quadruple and quintuple lines, compared to untransformed controls. We show that there was increased labelling of C_4 acids in both sets of plants compared to wild type, consistent with partial low-level function of a portion of the C_4 pathway.

Materials and Methods

Plant materials

Individual transgenic lines were generated overexpressing four of the core C_4 cycle enzymes required for a functional NADP-ME C_4 cycle (Supplementary Figure 1), *Zm*PEPC, *Zm*NADP-MDH, *Zm*NADP-ME and *Zm*PPDK. To express high levels of *Zm*PEPC (GRMZM2G083841), *Zm*PPDK (GRMZM2G306345), *Zm*NADP-MDH (GRMZM2G129513) and *Zm*NADP-ME (GRMZM2G085019) in rice, full-length genomic fragments encompassing the genes encoding these maize enzymes and their promoters were cloned into the pSC0 vector (GenBank, Accession no. KT365905; Lin *et al*. 2016). Generation of pSC0/*Zm*PEPC vector was previously described (Giuliani *et al.* 2019a). A pSC0/*Zm*PPDK vector containing a full-length genomic fragment was created by subcloning *Zm*PPDK from pIG121Hm/PPDK (a gift from Mitsue Miyao, NIAS, Japan; Matsuoka 1990) into pSC0. Gibson assembly (Giboson *et al*. 2009) was used to insert the gene into the pSC0 vector. The necessary amplicons from the pIG121Hm/PPDK and pSC0 templates were amplified using Primer I: 5'-ATGCTCAACACATGAGCGAAGGGCCCATGACCATGATTACGCCAAG, Primer II: 5'-TGTGCATGTCGCTAGGATCCGGTACCGAATGCTAGAGCAGCTTGA, Primer III: TCAAGCTGCTCTAGCATTCGGTACCGGATCCTAGCGACATGCACA, Primer IV: 5'-CTTGGCGTAATCATGGTCATGGGCCCTTCGCTCATGTGTTGAGCAT). The full-length genomic sequences of *Zm*NADP-ME and *Zm*NADP-MDH were amplified from bacterial artificial chromosomes sourced from BACPAC resources (Children's Hospital Oakland, California) (Coordinates CH201-14H23 for *Zm*NADP-ME and CH201-117G14 for *Zm*NADP-MDH) by PCR using primers (5'-ACGACGGCCAGTGCCAAGCTTCCCTTCCGTCAGCAGATTAGGCG and 5'-ATTATTATGGAGAAACTCGAGGCAACATGGTTCTGGACCGATTCAG for *Zm*NADP-MDH; 5'-

ACGACGGCCAGTGCCAAGCTTGGAATGACCACGAAATCGTCAAGCTAATCC and 5'- ATTATTATGGAGAAACTCGAGCTGTTACTGCTCTTTCCACTACTGAAGCAG for *Zm*NADP-ME) and subcloned into pSC0 vector. A binary vector with the hygromycin B resistance gene, pCAMBIA1300, was co-transformed with these vectors to allow for selection. The transformation of *indica* rice (*Oryza sativa* cv. IR64) was performed at the International Rice Research Institute (IRRI; Los Baños, Philippines) following a previously described method (Lin *et al*. 2016). In almost all cases, three independent single insertion homozygous transgenic lines with high transgene expression were selected for molecular and biochemical evaluation. However, for *Zm*NADP-ME, protein expression was only detected in a single transgenic line containing >6 copies of the overexpression construct and so this was the only line that could be taken forward. The line with highest protein expression for each gene was selected for stacking into a multi-transgenic single line through conventional crossing to create a quadruple cross line (PEPC/PPDK/MDH/ME) for investigation in the present study. The *Zm*PEPC and *Zm*NADP-MDH single transgenic lines were initially crossed to create a double transgene line that was then crossed with the *Zm*PPDK single transgenic line. This triple line (PEPC/PPDK/MDH) was then crossed with the *Zm*NADP-ME single transgenic line to produce a quadruple line (PEPC/PPDK/MDH/ME). A quintuple cross (PEPC/PPDK/MDH/ME/*gdch*) was then generated by crossing the quadruple F_2 line with a single *Osgdch* knockdown line (*gdch*-38) described by Lin *et al.* (2016). The presence of transgenes was determined by genomic PCR and protein accumulation by immunoblotting in each crossed line.

Plant growth

Plants were grown under natural light conditions in a screenhouse with a day/night temperature of 35/28 ± 3°C at the International Rice Research Institute (Los Baños, Philippines: 14° 10019.900N, 121° 15022.300E). Maximum irradiance was 2000 µmol photons m^{-2} s^{-1} on a sunny day. Plants were grown in 7-liter pots filled with soil from the IRRI upland farm.

171

172 *Immunoblotting*

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174 Leaf samples for soluble protein extraction were harvested from the fourth fully-
175 expanded leaf at the mid-tillering stage between 09:00 h and 11:00 h, and stored on ice
176 immediately. Leaves were homogenized to a fine powder using a nitrogen-cooled
177 mortar and pestle. Proteins were extracted and fractionated by SDS-PAGE as
178 described previously (Lin *et al.* 2016). Samples were loaded based on equal leaf area
179 (0.2364 mm^2 for *Zm*PEPC and *Zm*PPDK, and 2.364 mm^2 for *Zm*NADP-MDH, *Zm*NADP-
180 ME and *Os*GDCH). After electrophoresis, proteins were electroblotted onto a
181 polyvinylidene difluoride membrane and probed with rabbit antisera against *Zm*PEPC,
182 *Zm*NADP-MDH, *Zm*NADP-ME (all provided by Richard Leegood, University of Sheffield,
183 UK), *Zm*PPDK (provided by Chris Chastain, Minnesota State University, USA) and
184 *Os*GDCH protein (provided by Asaph Cousins, Washington State University, USA). The
185 dilutions of *Zm*PEPC, *Zm*PPDK, *Zm*NADP-MDH, *Zm*NADP-ME, and *Os*GDCH antisera
186 were 1:20,000, 1:20,000, 1:5,000, 1:2,000 and 1:100, respectively. A peroxidase-
187 conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich, USA;
188 https://www.sigmaaldrich.com/) was used at a dilution of 1:5,000 and immunoreactive
189 bands were visualized with ECL Western Blotting Detection Reagents (GE Healthcare,
190 UK; https://www.gelifesciences.com).

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192 *Immunolocalization*

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194 The middle portion of the seventh fully expanded leaf at the mid-tillering stage was
195 sampled between 09:00 h and 11:00 h and processed as described previously by Lin *et*
196 *al*. (2016). After fixation and cutting, the thin leaf sections were probed with the antisera
197 against *Zm*NADP-MDH, *Zm*NADP-ME, *Zm*PPDK and *Zm*PEPC at dilutions of 1:500,
198 1:25, 1:10 and 1:200, respectively. The secondary Alexa Fluor 488 goat anti-rabbit IgG

(Invitrogen, USA; https://www.thermofisher.com/ph/en/home/brands/invitrogen.html) antibody was used at a dilution of 1:200. The sections were visualized on a BX61 microscope fitted with a Disk Scanning Unit attachment microscope (Olympus, USA; https://www.olympus-global.com/) with fluorescence function under DAPI, RFP and GFP filters.

Enzyme activity measurement

Leaf samples were harvested between 09:00 h and 11:00 h from the youngest fully-expanded leaf of plants at the mid-tillering stage, and frozen immediately. Leaves were homogenized to a fine powder using a nitrogen-cooled mortar and pestle and extracted in 250 µL of buffer containing: 50 mM HEPES-KOH, pH7.4, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM Dithiothreitol, 1% (v/v) glycerol. After centrifugation at 10,000x*g* for 2 min at 4°C, the supernatant was collected for enzyme activity measurements. PEPC enzyme activity was assayed using a method modified from Meyer *et al*. (1988) and Ueno *et al*. (1997). The PEPC reaction mixture contained: 100 mM HEPES-NaOH, pH 7.5, 10 mM $MgCl_2$, 1 mM $NaHCO_3$, 5 mM G6P, 0.2 mM NADH, 12 unit/mL MDH (from pig heart; Roche Diagnostics, Basel, Switzerland; https://www.roche.com/), and the reaction was started by adding PEP to a final concentration of 4 mM. PPDK enzyme activity was assayed as described by Fukayama *et al*. (2001). NADP-MDH activity was determined by a method modified from Tscuhida *et al*. (2001). NADP-MDH reaction mixture contained: 50 mM HEPES-KOH, pH 8, 70 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.2 mM NADPH, and the reaction was started by adding OAA to a final concentration of 1 mM. NADP-ME activity was measured by a method modified from Tscuhida *et al*. (2001; protocol 1). The activities of PEPC, PPDK, NADP-MDH and NADP-ME were measured spectrophotometrically at 340 nm at 25°C, 30°C, 25°C and 25° C, respectively.

Gas exchange measurements

Leaf gas-exchange measurements were made using a Li-6400XT infrared gas analyzer (LI-COR Biosciences, USA; https://www.licor.com/) fitted with a standard 2 × 3 cm leaf chamber and a 6400-02B light source. Measurements were made at a constant airflow rate of 400 µmol s^{-1}, leaf temperature of 25°C, leaf-to-air vapor pressure deficit between 1.0 and 1.5 kPa and relative humidity of 60-65%. Data were acquired between 08:00 h and 13:00 h. Measurements were made from two youngest fully expanded leaves for each plant during the tillering stage. The mid-portions of leaves were acclimated in the cuvette for approximately 30 min before measurements were made. The response curves of the net CO_2 assimilation rate (*A*, µmol m^{-2} s^{-1}) to changing intercellular *p*CO_2 concentration (*Ci*, µmol CO_2 mol air^{-1}) were acquired by decreasing *Ca* (*p*CO_2 concentration in the cuvette) from 2000 down to 20 µmol CO_2 mol air^{-1} at a photosynthetic photon flux density (PPFD) of 1500 or 2000 µmol photon m^{-2} s^{-1}. The CO_2 compensation point (*Γ*) and maximum carboxylation efficiency (*CE*) were calculated from the intercept (Vogan *et al*. 2007) and slope (Wang *et al*. 2006) of the CO_2 response curves. Light response curves were acquired by increasing the PPFD from 0 to 2000 µmol photon m^{-2} s^{-1} at *Ca* 400 µbar. The quantum efficiency for CO_2 assimilation (φ) and respiration rates (R_d) were calculated from the slope and intercept of the light-response curves (PPFD < 100 µmol photons m^{-2} s^{-1}).

$^{13}CO_2$ pulse-labelling and quenching procedure

Carbon flux analysis was performed with a custom-built gas exchange freeze clamp apparatus (Supplementary Figure 2). Measurements were made from two youngest fully expanded leaves for each plant during the tillering stage. Two leaves of up to 22 cm in length were placed inside a gas exchange chamber (23.5 x 4.5 x 0.4 cm), the top was constructed from a piece of clear flexible plastic to allow light penetration and the bottom from a sheet of aluminum foil to accelerate cooling when freeze clamping. The foil and plastic were attached with foil tape to a three-sided aluminum frame to provide

rigidity. Two holes were drilled through the side of the frame to accommodate the air inlet and outlet tubes. A third hole on the end enabled thermocouples to be threaded through the frame to measure leaf and air temperature inside the labelling chamber. The chamber was then placed in a mounting frame allowing the leaf to be inserted prior to sealing the chamber with a foam gasket secured with bulldog clips. The mounting frame was positioned horizontally between two LED banks capable of providing illumination to the upper leaf surface of up to 1,000 µmol photons m^2 s^{-1}.

Air was drawn from outside the laboratory through a compressor. The air stream passed through an oil water separator and flow control valve into a copper coil placed in an ice bath for cooling. The air stream could be directed into the leaf chamber or by-passed into a CO_2 conditioning unit. In the latter, CO_2 could be removed from the air with soda lime and then optionally enriched with $^{13}CO_2$ gas (300 ppm) prepared by mixing $NaH^{13}CO_3$ (Sigma-Aldrich, USA; https://www.sigmaaldrich.com/) with 2-hydroxypropanoic acid (lactic acid). The flow of air passing over the leaf (3 ml/min) was adjusted with a flow controller, and the CO_2 concentration of the incoming and outgoing air streams measured with two CO_2 analyzers (WMA-5, PP-Systems, USA; https://ppsystems.com/). The humidity of the air inside the chamber was maintained at ~60% with the addition of water to the soda lime chamber or reduced by passing air through a chamber of silica gel.

The leaf chamber was mounted on the stand in such a way that the plane of the leaf was halfway between two pneumatically operated aluminum bars. These were cooled with liquid nitrogen, and when released they clamped together fitting inside the aluminum chamber frame, very rapidly freezing the leaf. A fan was mounted horizontally to the bars to blow the fog from the liquid nitrogen away from the chamber to ensure there was minimal disruption to the environment before freezing occurred. The lower bar was positioned in such a way as to push the chamber up on closure. The cold temperatures and force of the bars closing meant the chamber disintegrated enabling the leaf to be removed with tweezers and placed in a liquid nitrogen bath for 10 s before subsequent storage at -80 °C. To perform $^{13}CO_2$ pulse-labelling, leaves were acclimated at steady-state conditions prior to scrubbing CO_2 from the incoming air stream, then

subjected to $^{13}CO_2$ enriched air for a duration of 0 and 60 s and metabolic activity was quenched at these time points by freeze clamping the leaves as above. Freeze-quenched tissue was ground into a fine powder by mortar and pestle in liquid nitrogen. Finely-ground leaf tissues were freeze-dried for 3 days and placed into sealed tubes. The sealed tubes containing finely-ground lyophilized leaf tissue samples were shipped to Max Planck Institute of Molecular Plant Physiology, Germany for the metabolite analyses.

Metabolite analyses and calculation of total pool size, enrichment and isotopomer distribution

Aliquots (3 or 5 mg) of finely-ground lyophilized rice leaf tissue were extracted with chloroform-methanol as described in Arrivault *et al.,* (2017), and the lyophilized extracts were resuspended in 300 µL or 600 µL purified (Millipore, USA) water, respectively. Isotopomers were measured by reverse-phase LC-MS/MS (malate, aspartate, 3PGA, PEP, citrate+isocitrate; Arrivault *et al.* 2017; n.b. citrate & isocitrate were not resolved using this method) and anion-exchange LC-MS/MS (malate, PEP, citrate; Lunn *et al*. 2006 with modifications as described in Figueroa *et al.* 2016). Total amounts of malate, aspartate, citrate+isocitrate and citrate were calculated by summing isotopomers. The total amounts of 3PGA and PEP were determined enzymatically in trichloroacetic acid extracts using a Sigma-22 dual-wavelength photometer (Merlo *et al.* 1993), with PEP being measured in freshly prepared extracts. ^{13}C enrichment and relative isotopomer distribution were calculated as in Szecowka *et al*. (2013).

Statistical analysis

Statistical analysis for all experiments was performed in R version 3.0.0 (The R Foundation for Statistical Computing, Vienna, Austria) using a one-way analysis of

variance (ANOVA) and a Tukey post-hoc test or a Student's t-test with a *p*-value of <0.05.

Results

Overexpression of C_4 cycle genes in *Oryza sativa*

Immunoblotting of F_2 generation transgenic plants showed that the protein of the correct size for *Zm*PEPC, *Zm*NADP-MDH, *Zm*NADP-ME and *Zm*PPDK was stably expressed in both the quadruple and quintuple cross rice lines, with *Os*GDCH protein almost undetectable in the quintuple cross (Figure 1). In all lines, protein abundance was lower than that of wild-type maize plants. We next sought to determine whether these proteins were localized to the correct cell type and subcellular compartment. Immunolocalization analysis of the quadruple cross line revealed that *Zm*PEPC was localized to the cytosol of MCs similar to the single *Zm*PEPC transgenic line (Giuliani *et al*. 2019b) and *Zm*PPDK, was localized to the chloroplast in both BSCs and MCs (Supplementary Figure 3). The *Zm*NADP-MDH and *Zm*NADP-ME antisera cross-reacted with native protein in wild-type rice and so it was not possible to distinguish protein encoded by the endogenous rice gene from that encoded by the maize transgene. Overexpression of these enzymes conferred enhanced enzyme activity (Supplementary Table 1). In the quadruple cross line, PEPC activity was 18.4-fold higher compared to wild-type rice plants, PPDK 5.8-fold higher, NADP-MDH 9.9-fold and NADP-ME 4.1-fold.

Phenotypic and photosynthetic perturbations associated with overexpression of C_4 cycle genes

Given that protein levels and activities of all four introduced C_4 enzymes were enhanced compared to wild-type plants, we investigated whether this affected growth and photosynthesis. None of the crossed lines consistently showed altered chlorophyll content (Figure 2A). Tiller number in the quintuple cross lines (Figure 2B) and plant height in both quadruple and quintuple crosses (Figure 2C) were significantly reduced.

Phenotypic perturbations were most marked in the quintuple cross lines (Supplementary Figure 4), although the plants still developed and flowered at the same time with wild-type plants. These growth perturbations were not observed in the single C_4 gene transgenic lines of *Zm*PEPC, *Zm*PPDK, *Zm*NADP-MDH and *Zm*NADP-ME (Giuliani et al. 2019) but were observed in the single *Osgdch* knockdown line (Lin *et al*. 2016).

To investigate whether overexpression of C_4 genes impacted photosynthesis, the response of net CO_2 assimilation rate (*A*) to CO_2 concentration under photorespiratory conditions (21% O_2) was measured. In the quadruple cross line there were no differences in CO_2 assimilation (Figure 3A, C), Γ, CE, R_d or Φ compared to wild-type plants (Table 1). These results suggested that enhanced activity of C_4 cycle enzymes in a cell-specific manner does not significantly affect leaf level photosynthetic gas-exchange and that the phenotypic perturbations are not associated with reduced CO_2 assimilation.

For the quintuple cross, the CO_2 assimilation rate in response to increasing intercellular CO_2 concentrations (*Ci*) was reduced, most notably at lower CO_2 concentrations (<700 µmol CO_2 mol^{-1}, Figure 3B). Under non-photorespiratory conditions (2% O_2, Figure 3B) CO_2 assimilation rates were similar to wild-type plants. Consistent with this, the quintuple cross had a significantly higher Γ under high photorespiratory conditions but not under low photorespiratory conditions (Table 1). In response to changes in photon flux density, photosynthesis was saturated at lower light levels than in wild-type plants (400 µmol photons m^{-2} s^{-1} versus 1750 µmol photons m^{-2} s^{-1}, respectively, Figure 3D) with significantly lower Φ and higher R_d than wild-type plants (Table 1).

To investigate these photosynthetic responses for the quintuple cross line in more detail, CO_2 responses were measured under conditions conducive to low and high rates of photorespiration. Under low light (400 µmol photons m^{-2} s^{-1}) and high CO_2 (2000 µmol CO_2 mol air^{-1}), conditions conducive to low photorespiration, CO_2 responses of the quintuple cross line were similar to wild-type plants (Supplementary Figure 5A, D). Under high light (1500 µmol photons m^{-2} s^{-1}) and low CO_2 (400 µmol CO_2 mol air^{-1}), conditions conducive to high photorespiration, CO_2 assimilation was lower in the quintuple cross (Supplementary Figure 5B, C). Correspondingly, Γ was higher, and

carboxylation efficiency (*CE*) lower under conditions conductive to high rates of photorespiration but not under non-photorespiratory conditions (Supplementary Table 2). This is consistent with the photorespiratory-deficient phenotype observed in the single *Osgdch* knockdown lines (Lin *et al*. 2016).

Increased incorporation of ^{13}C into C_4 acids associated with overexpression of C_4 cycle enzymes

We performed experiments to measure the flux of $^{13}CO_2$ through photosynthetic metabolism to investigate whether there was partial functionality of a C_4 pathway in these plants. There was significantly more incorporation of ^{13}C into malate in the quadruple and quintuple cross lines than in wild-type plants (Figure 4, Supplementary Figure 6), with the m_1 isotopomer being more abundant than the other ^{13}C-labelled isotopomers (Table 2), consistent with increased fixation of $^{13}CO_2$ via PEPC in the transgenic lines expressing the maize PEPC enzyme. Labelling of aspartate was also significantly higher than wild-type in the quintuple line (Figure 4, Supplementary Figure 6), with the m_1 isotopomer being more abundant that m_2-m_4 isotopomers (Table 2), indicating additional $^{13}CO_2$ fixation into oxaloacetate by maize PEPC and conversion to aspartate by endogenous rice aspartate aminotransferase activity. There was almost complete labelling of the 3PGA pool in wild-type plants after a 60 s pulse, and similarly high levels of labelling of 3PGA were observed in the quadruple and quintuple lines (Figure 4, Supplementary Figure 6). There was also substantial labelling of PEP after a 60 s pulse, with no significant differences between the transgenic lines and wild-type plants (Figure 4, Supplementary Figure 6). There was almost no labelling of citrate and isocitrate in wild-type plants and the quadruple line (Figure 4). In contrast, the enrichment of ^{13}C in citrate and isocitrate in the quintuple line was 10-fold higher than in wild-type plants.

Discussion

We have previously shown that overproduction of individual C_4 enzymes in rice has no consistent effect on CO_2 assimilation or plant growth (Giuliani *et al*. 2019b). The exception to this was the transgenic line overexpressing *Zm*NADP-ME which exhibited a small decrease in plant height and reduced maximal photosynthetic rate at high CO_2. Previous attempts to overproduce *Zm*NADP-ME in rice have led to increased photoinhibition of photosynthesis, leaf chlorophyll bleaching and serious stunting attributed to an increase in the NADPH/$NADP^+$ ratio in the chloroplast stroma due to the exchange with 2-oxoglutarate involved in photorespiration (Tsuchida *et al.* 2001). Severe phenotypic effects were not observed in our *Zm*NADP-ME line; however, we were only able to advance a single line containing 6 copies of the construct in which protein accumulation was higher than in our rice control, but still only around 10% of the activity found in maize. In contrast, the experiments of Tsuchida *et al.* (2001) used a rice chlorophyll a/b binging protein (*cab*) promoter allowed for high level but not cell specific expression, and activities of up to 60% of maize levels were achieved, leading to a much more severe phenotype. Overproduction of all four targeted C_4 enzymes in a single plant led to a slight decrease in tiller number and plant height but otherwise growth and photosynthesis were unaffected. These results are consistent with previous reports of engineering a single-cell C_4 pathway in rice (Taniguchi *et al*. 2008). A quintuple cross that combined over-expression of the four C_4 enzymes, *Zm*PEPC, *Zm*NADP-MDH, *Zm*NADP-ME and *Zm*PPDK with knockdown of the native rice *Os*GDCH, thereby compromising the photorespiratory pathway, led to further reductions in tiller number and plant height. A strong negative effect on photosynthesis was also observed in the quintuple cross consistent with the photorespiratory-deficient phenotype of the single *Osgdch* knockdown line (Lin *et al*. 2016).

Our results show that *Zm*PEPC is catalytically active *in vivo* when expressed in combination with other C_4 enzymes in rice, and substantially increases the fixation of CO_2 into C_4 acids. This is in contrast to published radiolabelling studies of rice expressing *Zm*PEPC alone (Fukayama *et al.* 2003; Miyao *et al.* 2011) in which there was no increase in incorporation of labelled carbon into C_4 acids, despite the extractable activity of PEPC in these plants approaching or exceeding maize levels. Despite strong evidence for operation of the C_4 pathway in our transgenic lines up to the

point of malate production, there was no evidence for the regeneration of PEP via the rest of the C_4 cycle. The labelling of PEP at a similar level in all three genotypes, wild-type, quadruple and quintuple crosses, suggests that rather than being produced by a functional C_4 cycle, PEP is being produced from 3PGA via 2PGA catalyzed by phosphoglyceromutase and enolase (Furbank and Leegood 1984), consistent with the majority of CO_2 still being fixed via Rubisco in C_3 photosynthesis rather than through the operation of a complete C_4 cycle.

The very low incorporation of ^{13}C into citrate and isocitrate in wild-type rice plants is consistent with previous studies (Tcherkez *et al.* 2009; Szecowka *et al.* 2013) indicating little flux of carbon into the tricarboxylic acid (TCA) cycle via mitochondrial pyruvate dehydrogenase (mPDH) in the light, due to deactivation of the mPDH by phosphorylation (Randall *et al.* 1996; Tovar-Méndez *et al.* 2003). The increased labelling of citrate in the quintuple cross line suggests that the mPDH is more active in the light in this line, potentially leading to respiration of C_4 acids via the TCA cycle, which would be deleterious for C_4 photosynthetic flux. This might be due to lower rates of photorespiration leading to less photophosphrylation of PDH (Tovar-Méndez *et al.* 2003). Further, increased levels of pyruvate in the mitochondria (from decarboxylation of malate by NAD-malic enzyme), can inhibit the mPDK kinase (Schuller & Randall 1990). We propose that there is a modified regulation of the TCA cycle to avoid wasteful respiration of C_4 acids, and that such modification might have been needed for the evolution of an efficient C_4 photosynthetic pathway.

Evidence that *Zm*PEPC can be localized to the cytosol of MCs (Giuliani *et al*. 2019b) and is catalytically active, leading to the fixation of CO_2 into C_4 acids provides important evidence in support of installing a fully functional C_4 photosynthetic pathway into rice. However, absolute quantification of flux into and through C_4 acids would require further pulse-chase labelling studies and may prove difficult with the low rates of labelling relative to C_3 photosynthetic fixation obtained in the current transgenic lines.

Cell specificity of expression of the C_4 enzymes introduced into the rice lines shown here remains an important issue. We have been able to achieve localization of *Zm*PPDK to rice MCs and additional localization to the chloroplast of rice BSCs by the

use of a native *Zm*PPDK promoter. This result is consistent with *Zm*PPDK expression in both MCs and BSCs in maize (Sheen and Bogorad 1987; Miyao et al. 2011). However, we were unable to conclude with confidence whether there was correct localization of *Zm*NADP-ME and *Zm*NADP-MDH in BSCs and MCs, respectively. When used to drive *β-GLUCURONIDASE* (*GUS*) expression, the promoter used for *Zm*NADP-ME expression was shown to lead to GUS accumulation in both BSCs and MCs in rice (Nomura *et al*. 2005), suggesting that exclusive localization of NADP-ME in the BSCs of our plants is unlikely. Incorrect or partial localization of enzymes would potentially limit the operation of a C_4 cycle (Miyao *et al*. 2011), and higher-level expression of *Zm*NADP-ME than that achieved in our lines, particularly if mis-expressed in the M cells, may produce quite deleterious phenotypes (Tsuchida *et al.* 2001).

In addition to high level, cell specific expression of C_4 cycle enzymes in rice, fully functional C_4 photosynthetic biochemistry requires appropriate enzyme regulation in the environment of a rice leaf cell (Burnell and Hatch 1985; Chastain *et al*. 1997). For example, the activity of C_4 specific PPDK is regulated in the light through protein phosphorylation by the PPDK regulatory protein (Burnell and Hatch 1985). Similarly NADP-MDH is regulated by light through the thioredoxin cascade (Miginiac-Maslow *et al*. 2000). Both enzymes are regulated in the same manner even when expressed within C_3 leaves (Fukayama *et al*. 2001; Taniguchi *et al*. 2008). A recent study has shown that C_4 NADP-ME is also regulated in the light by reversible phosphorylation at Ser419 which is involved in the binding of NADP at the active site (Boydiloya et al. 2019). In contrast, PEPC is regulated by both metabolite effectors and reversible phosphorylation, but the mechanisms of regulation in C_3 and C_4 leaves are different (Vidal and Chollet 1997). Indeed, Fukayama *et al.* (2003) observed inappropriate phosphorylation of PEPC in their transgenic rice lines and proposed this as a reason for lack of labeling of C_4 acids in the light. The regulatory mechanisms for other enzymes are less well understood. It is unclear at present whether enzyme levels per se or enzyme regulation in our rice transgenic lines, or both, is limiting C_4 flux.

In addition to the metabolic enzymes of the C_4 pathway, there is a need to identify and overproduce the metabolite transporters required to support C_4 photosynthesis (Weber

and von Caemmerer 2010; Ermakova *et al*. 2019). While many of the necessary transporters may be present at low levels in C_3 chloroplasts (Weber and von Caemmerer 2010), their activities might be insufficient to mediate the greater fluxes across the chloroplast envelope that are required for operation of a C_4 cycle.

A plethora of other changes are required to support a fully functional C_4 pathway in rice. This includes, but is not limited to, engineering the correct leaf anatomy (Hattersley and Watson 1975, Dengler *et al*. 1994; Muhaidat *et al*. 2007; Dengler and Taylor 2000) and morphological specializations such as increased vein density (Sedelnikova *et al*. 2018). In addition, thought must be given to the photosynthetic functionalisation of the BSCs of rice, which contain a large central vacuole, with very few mitochondria, peroxisomes or chloroplasts (Sage and Sage 2009). Where chloroplasts do occur, they are smaller than those in MCs. In addition, MCs of rice are highly lobed to assist with photorespiratory CO_2 scavenging (Sage and Sage 2009); whereas the MCs of C_4 species are not. Increasing chloroplast number and volume in the BSCs will no doubt be important for achieving C_4 photosynthesis in rice (Chonan 1970, 1978; Dengler *et al*. 1994; Ueno *et al*. 2006; Wang *et al.* 2017). Insufficient chloroplast volume in the BSCs of rice may have led to limitations in C_4 acid decarboxylation in the transgenic lines described here. Other modifications such as cross-sectional area of the BSCs, modifying the cell wall properties for diffusion of CO_2 (von Caemmerer and Furbank 2003) and increasing plasmodesmatal frequency at the BSC / MC interface to support metabolite diffusion may be necessary (Ermakova *et al*. 2019). The genetic regulators of many of these changes are not known, and so future goals include identification and incorporation of necessary genes for anatomical modifications into a version of the current biochemical prototype, with the ultimate goal of engineering an efficient C_4 pathway in rice.

Supplementary Materials

Supplementary Dataset A. Isotopomer and metabolite amounts, 13C enrichments and relative isotopomer abundances of malate, aspartate, 3PGA, PEP and citrate+isocitrate in wild-type, quadruple and quintuple lines.

Supplementary Dataset B. Isotopomer and metabolite amounts, 13C enrichments and relative isotopomer abundances of malate, aspartate, 3PGA, PEP and citrate in wild-type and quintuple lines.

Author Contributions

HCL, SA, RAC, JEL, MS, RTF and WPQ designed the experiments together. HCL provided all the plant materials. HCL and EB performed enzyme activity assay, immunobloting, immunolocalization and gas exchange measurements. WPQ, RTF, MS, JEL and RAC designed the gas exchange freeze clamp apparatus. SA performed metabolite analysis. HCL, SA, RAC and WPQ wrote the manuscript. SC and JMH designed constructs. SK performed plant transformation. HCL and RAC performed the $^{13}CO_2$ labelling experiment.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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References

Arrivault S., Obata T., Szecówka M., Mengin V., Guenther M., Hoehne M., et al. (2017). Metabolite pools and carbon flow during C_4 photosynthesis in maize:$^{13}CO_2$ labeling kinetics and cell type fractionation. J. Exp. Bot. 68, 283-298. doi: 10.1093/jxb/erw414

Bovdilova A., Alexandre B.M., Höppner A., Luís I.M. , Alvarez C.E., Bickel D., et al. (2019). Posttranslational Modification of the NADP-Malic Enzyme Involved in C_4 Photosynthesis Modulates the Enzymatic Activity during the Day. Plant Cell. 31, 2525-2539. doi: 10.1105/tpc.19.00406

Burnell J.N., Hatch M.D. (1985). Light-dark modulation of leaf pyruvate, Pi dikinase. Trends Biochem. Sci. 10, 288-291. doi:10.1016/0968-0004(85)90090-8

von Caemmerer S., Furbank R.T. (2003). The C_4 pathway: an efficient CO_2 pump. Photosynth. Res. 77, 191-207. doi: 10.1023/A:1025830019591

von Caemmerer S., Quick W.P., Furbank R.T. (2012). The development of C_4 rice: current progress and future challenges. Science 336, 1671-1672. doi: 10.1126/science.1220177

Chastain C.J., Lee M.E., Moorman M.A., Shameekumar P., Chollet R. (1997). Site-directed mutagenesis of maize recombinant C_4-pyruvate, orthophosphate dikinase at the phosphorylatable target threonine residue. FEBS Letters 413, 169-173. doi: 10.1016/S0014-5793(97)00884-3

Chonan N. (1970). Studies on the photosynthetic tissues in the leaves of cereal crops. V. Comparison of the mesophyll structure among seedling leaves of cereal crops. Proc. Crop Sci. Soc. Jpn. 39, 418-425. doi: 10.1626/jcs.39.418

Chonan N. (1978). Comparative anatomy of mesophyll among the leaves of gramineous crops. Jpn. Agr. Res. Q. 12, 128-131.

Dengler N.G., Dengler R.E., Donnelly P.M., Hattersley P.W. (1994). Quantitative leaf anatomy of C_3 and C_4 grasses (*Poaceae*)—bundle- sheath and mesophyll surface-area relationships. Ann. Bot. 73, 241-255. doi: 10.1006/anbo.1994.1029

Dengler N., Taylor W.C. (2000). Developmental aspects of C_4 photosynthesis. In: Photosynthesis: physiology and metabolism, eds, R.C. Leegood, T.D. Sharkey, S. von

573 Caemmerer (Dordrecht, The Netherlands: Springer), 471-495. doi: 10.1007/0-306-
574 48137-5_20

575 Ermakova M., Danila F.R., Furbank R.T., von Caemmerer S. (2019). On the road to C_4
576 rice: advances and perspectives. Plant J. 101, 940-950. doi: 10.1111/tpj.14562

577 Figueroa C.M., Feil R., Ishihara H., Watanabe M., Kölling K., Krause U., et al. (2016).
578 Trehalose 6-phosphate coordinates organic and amino acid metabolism with carbon
579 availability. Plant J. 85, 410-423. doi: 10.1111/tpj.13114

580 Fukayama H., Tsuchida H., Agarie S., Nomure M., Onodera H., Ono K., et al. (2001).
581 Significant accumulation of C_4-specific pyruvate, orthophosphate dikinase in a C_3 plant,
582 rice. Plant Physiol. 127, 1136-1146. doi: 10.1104/pp.010641

583 Fukayama H., Hatch M.D., Tamai T., Tsuchida H., Sudoh S., Furbank R.T., et al.
584 (2003). Activity regulation and physiological impacts of maize C_4-specific
585 phosphoenolpyruvate carboxylase overproduced in transgenic rice plants. Photosynth.
586 Res. 77, 227-239. doi: 10.1023/A:1025861431886

587 Furbank R.T., Leegood R.C. (1984). Carbon metabolism and gas exchange in leaves of
588 *Zea mays* L.. Planta 162, 457-462. doi: 10.1007/BF00393458

589 Giuliani R., Karki S., Covshoff S., Lin H.C., Coe R.A., Koteyeva N.K., et al. (2019a).
590 Knockdown of glycine decarboxylase complex alters photorespiratory carbon isotope
591 fractionation in *Oryza sativa* leaves. J. Exp. Bot. 70, 27773-27786. doi:
592 10.1093/jxb/erz083

593 Giuliani R., Karki S., Covshoff S., Lin H.C., Coe R.A., Koteyeva N.K., et al. (2019b).
594 Transgenic maize phosphoenolpyruvate carboxylase alters leaf-atmosphere CO_2 and
595 $^{13}CO_2$ exchange in *Oryza sativa*. Photosynth. Res. 142, 153-167. doi: 10.1007/s11120-
596 019-00655-4

597 Ghannoum O., Evans J.R., von Caemmerer S. (2011). Nitrogen and water use
598 efficiency of C_4 plants, In: C_4 Photosynthesis and Related CO_2 Concentrating
599 Mechanism, eds, A.S. Raghavendra, R.F. Sage (Dordrecht, The Netherlands: Springer),
600 129-146.

Gibson D.G., Young L., Chuang R.Y., Venter J.C., Hutchison C.A. 3rd, Smith H.O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343-345. doi: 10.1038/nmeth.1318

Hatch M.D. (1971). The C_4 pathway of photosynthesis. Biochem. J. 125, 425-432. doi: 10.1042/bj1250425

Hatch M.D., Kagawa T., Craig S. (1975). Subdivision of C_4-pathway species based on differing C_4 acid decarboxylating systems and ultrastructural features. Aust. J. of Plant Physiol. 2, 111-128. doi: 10.1071/PP9750111

Hatch M.D. (1987). C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochim. Biophys. Acta 895, 81-106. doi: 10.1016/S0304-4173(87)80009-5

Hatch M.D., Slack C.R., Johnson H.S. (1967). Further studies on a new pathway of photosynthetic carbon dioxide fixation in sugar-cane and its occurrence in other plant species. Biochem. J. 102, 417-422. doi: 10.1042/bj1020417

Hattersley P.W., Watson L. (1975). Anatomical parameters for predicting photosynthetic pathways of grass leaves: The 'maximum lateral cell count' and the 'maximum cells distant count'. Phytomorph. 25, 325–333.

Hibberd J.M., Sheehy J.E., Langdale J.A. (2008). Using C_4 photosynthesis to increase the yield of rice-rationale and feasibility. Curr. Opin. Plant. Biol. 11, 228-231. doi: 10.1016/j.pbi.2007.11.002

Kajala K., Covshoff S., Karki S., Woodfield H., Tolley B.J., Dionora M.J.A., et al. (2011). Strategies for engineering a two-celled C_4 photosynthetic pathway into rice. J. Exp. Bot. 62, 3001-3010. doi: 10.1093/jxb/err022

Lin H.C., Karki S., Coe R.A., Bagha S., Khoshravesh R., Balahadia C.P., et al. (2016). Targeted knockdown of *GDCH* in rice leads to a photorespiratory deficient phenotype useful as building block for C_4 rice. Plant Cell Physiol. 57, 919-932. doi: 10.1093/pcp/pcw033

628 Lunn J.E., Feil R., Hendriks J.H., Gibon Y., Morcuende R., Osuna D., et al. (2006).
629 Sugar-induced increases in trehalose 6−phosphate are correlated with redox activation
630 of ADP-glucose pyrophosphorylase and higher rates of starch synthesis in Arabidopsis
631 thaliana. Biochem. J. 397, 139-148. doi: 10.1042/BJ20060083

632 Matsuoka M., Kyozuka J., Shimamoto K., Kano-Murakami Y. (1994). The promoters of
633 two carboxylases in a C_4 plant (maize) direct cell-specific, light-regulated expression in
634 a C_3 plant (rice). Plant J. 6, 311-319. doi: 10.1046/j.1365-313x.1994.06030311.x

635 Matsuoka M. (1990). Structure, genetic mapping, and expression of the gene for
636 pyruvate, orthophosphate dikinase from maize. J. Biol. Chem. 265, 16772-16777.

637 Meyer C.R., Rustin P., Wedding R.T. (1988). A simple and accurate spectrophotometric
638 assay for phosphoenolpyruvate carboxylase activity. Plant Physiol. 86, 325-328. doi:
639 10.1104/pp.86.2.325

640 Merlo L., Geigenberger P., Hajirezaei M., Stitt M. (1993). Changes of carbohydrates,
641 metabolites and enzyme activities in potato tubers during development, and within a
642 single tuber along astolon-apexgradient. J. Plant Physiol. 142, 392-402. doi:
643 10.1016/S0176-1617(11)81243-5

644 Miginiac-Maslow M., Johansson K., Ruelland E., Issakidis-Bourguet E., Schepens I.,
645 Goyer A., Lemaire-Chamley M, Jacquot JP, Le Maréchal P, Deccotignies P. et al.
646 (2000). Light- activation of NADP-malate dehydrogenase: a highly controlled process for
647 an optimized function. Physiol. Plant. 110, 322-329. doi: 10.1111/j.1399-
648 3054.2000.1100306.x

649 Miyao M. (2003). Molecular evolution and genetic engineering of C_4 photosynthetic
650 enzymes. J. Exp. Bot. 54, 179-189. doi: 10.1093/jxb/erg026

651 Miyao M., Masumoto C., Miyazawa S.I., Fukayama H. (2011). Lessons from
652 engineering a single-cell C_4 photosynthetic pathway into rice. J. Exp. Bot. 62, 3021-
653 3029. doi: 10.1093/jxb/err023

654 Muhaidat R., Sage R.F., Dengler N.G. (2007). Diversity of Kranz anatomy and
655 biochemistry in C_4 Eudicots. Am. J. Bot. 94, 362-381. doi: 10.3732/ajb.94.3.362

Nomura M., Higuchi T., Ishida Y., Ohta S., Komari T., Imaizumi N., et al. (2005). Differential expression pattern of C_4 bundle sheath expression genes in rice, a C_3 plant. Plant and Cell Physiol. 46, 754-61. doi: 10.1093/pcp/pci078

Randall D.D., Miernyk J.A., David N.R., Gemel J., Luethy M.H. (1996). Regulation of leaf mitochondrial pyruvate dehydrogenase complex activity by reversible phosphorylation. In: Protein Phosphorylation in Plants, eds, P.R. Shewry, N.G. Halford, R. Hooley (Clarendon, UK: Oxford Press), 87-103.

Ray D.K., Mueller N.D., West P.C., Foley J.A. (2013). Yield trends are insufficient to double global crop production by 2050. PLoS ONE 8: e66428. doi: 10.1371/journal.pone.0066428

Sage R.F. (2004). The evolution of C_4 photosynthesis. New Phytol. 161, 341-370. doi: 10.1111/j.1469-8137.2004.00974.x

Sage T.L., Sage R.F. (2009). The functional anatomy of rice leaves: implications for refixation of photorespiratory CO_2 and efforts to engineer C_4 photosynthesis into rice. Plant Cell Physiol. 50, 756-772. doi: 10.1093/pcp/pcp033

Sage T.L., Sage R.F., Kocacinar F. (2012). Photorespiration and the evolution of C_4 photosynthesis. Annu. Rev. Plant Biol. 63, 19-47. doi: 10.1146/annurev-arplant-042811-105511

Schuller K.A., Randall D.D. (1990). Mechanism of pyruvate inhibition of plant pyruvate dehydrogenase kinase and synergism with ADP. Arch. Biochem. Biophys. 278, 211-216. doi: 10.1016/0003-9861(90)90250-3

Sedelnikova O.V., Hughes T.E., Langdale J.A. (2018). Understanding the Genetic Basis of C_4 Kranz Anatomy with a View to Engineering C_3 Crops. Annu. Rev. Genet. 52, 249-270. doi: 10.1146/annurev-genet-120417-031217

Sheen J.Y., Bogorad L. (1987). Differential expression of C_4 pathway genes in mesophyll and bundle sheath cells of greening maize leaves. J. Biol. Chem. 262, 11726-30.

683 Szecowka M., Heise R., Tohge T., Nunes-Nesi A., Vosloh D., Huege J., et al. (2013).
684 Metabolic fluxes in an illuminated Arabidopsis rosette. Plant Cell 25, 694-714. doi:
685 10.1105/tpc.112.106989

686 Taniguchi Y., Ohkawa H., Masumoto C., Fukuda T., Tesshu T., Lee K., et al. (2008).
687 Overproduction of C_4 photosynthetic enzymes in transgenic rice plants: an approach to
688 introduce the C_4-like photosynthetic pathway into rice. J. Exp. Bot. 59, 1799-1809. doi:
689 10.1093/jxb/ern016

690 Tcherkez G., Mahé A., Gauthier P., Mauve C., Gout E., Bligny R., et al. (2009). In folio
691 respiratory fluxomics revealed by ^{13}C isotopic labeling and H/D isotope effects highlight
692 the noncyclic nature of the tricarboxylic acid 'cycle' in illuminated leaves. Plant Physiol.
693 151, 620-630. doi: 10.1104/pp.109.142976

694 Tovar-Méndez A., Miernyk J.A., Randall D.D. (2003). Regulation of pyruvate
695 dehydrogenase complex activity in plant cells. Eur. J. Biochem. 270, 1043-1049. doi:
696 10.1046/j.1432-1033.2003.03469.x

697 Tsuchida H., Tamai T., Fukayama H., Agarie S., Nomura M., Onodera H., et al. (2001).
698 High level expression of C_4-specific NADP-malic enzyme in leaves and impairment of
699 photoautotrophic growth of a C_3 plant, rice. Plant Cell Physiol. 42, 138-145. doi:
700 10.1093/pcp/pce013

701 Ueno O., Kawano Y., Wakayama M., Takeda T. (2006). Leaf vascular systems in C_3
702 and C_4 grasses: a two-dimensional analysis. Annu. Bot. 97, 611-621. doi:
703 10.1093/aob/mcl010

704 Ueno Y., Hata S., Izui K. (1997). Regulatory phosphorylation of plant
705 phosphoenolpyruvate carboxylase: role of a conserved basic residue upstream of the
706 phosphorylation site. FEBS Letter 417, 57-60. doi: 10.1016/S0014-5793(97)01254-4

707 Vogan P.J., Frohlich M.W., Sage R.F. (2007). The functional significance of C_3-C_4
708 intermediate traits in *Heliotropium* L (Boraginaceae): gas exchange perspectives. Plant
709 Cell Environ. 30, 1337-1345. doi: 10.1111/j.1365-3040.2007.01706.x

Vidal J., Chollet R. (1997). Regulatory phosphorylation of C_4 PEP carboxylase. Trends Plant Sci. 2, 230-237. doi: 10.1016/S1360-1385(97)89548-9

Wang Q., Zhang Q., Fan D., Lu C. (2006). Photosynthetic light and CO_2 utilization and C_4 traits of two novel super-rice hybrids. J. Plant Physiol. 163, 529-537. doi: 10.1016/j.jplph.2005.04.035

Wang P., Khoshravesh R., Karki S., Tapia R., Balahadia C.P., Bandyopadhyay A., et al. (2017). Re-creation of a key step in the evolutionary switch from C_3 to C_4 leaf anatomy. Curr. Biol. 27, 3278-3287. doi: 10.1016/j.cub.2017.09.040

Weber A.P., von Caemmerer S. (2010). Plastid transport and metabolism of C_3 and C_4 plants — comparative analysis and possible biotechnological exploitation. Curr. Opin. in Plant Biol. 13, 256-264. doi: 10.1016/j.pbi.2010.01.007