Transgenic maize phosphoenolpyruvate carboxylase alters leaf–atmosphere CO₂ and ¹³CO₂ exchanges in *Oryza sativa*

Rita Giuliani¹ · Shanta Karki² · Sarah Covshoff³ · Hsiang-Chun Lin² · Robert A. Coe² · Nuria K. Koteyeva⁴ · Marc A. Evans⁵ · W. Paul Quick^{2,6} · Susanne von Caemmerer⁷ · Robert T. Furbank⁷ · Julian M. Hibberd³ · Gerald E. Edwards¹ · Asaph B. Cousins¹

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Abstract

The engineering process of C₄ photosynthesis into C₃ plants requires an increased activity of phosphoenolpyruvate carboxylase (PEPC) in the cytosol of leaf mesophyll cells. The literature varies on the physiological effect of transgenic maize (*Zea mays*) PEPC (*ZmPEPC*) leaf expression in *Oryza sativa* (rice). Therefore, to address this issue, leaf–atmosphere CO₂ and ¹³CO₂ exchanges were measured, both in the light (at atmospheric O₂ partial pressure of 1.84 kPa and at different CO₂ levels) and in the dark, in transgenic rice expressing *ZmPEPC* and wild-type (WT) plants. The in vitro PEPC activity was 25 times higher in the *PEPC* overexpressing (*PEPC*-OE) plants (~20% of maize) compared to the negligible activity in WT. In the *PEPC*-OE plants, the estimated fraction of carboxylation by PEPC (β) was ~6% and leaf net biochemical discrimination against ¹³CO₂ (Δ_{bio}) was ~2‰ lower than in WT. However, there were no differences in leaf net CO₂ assimilation rates (*A*) between genotypes, while the leaf dark respiration rates (*R_d*) over three hours after light–dark transition were enhanced (~30%) and with a higher ¹³C composition ($\delta^{13}\text{C}_{\text{Rd}}$) in the *PEPC*-OE plants compared to WT. These data indicate that *ZmPEPC* in the *PEPC*-OE rice plants contributes to leaf carbon metabolism in both the light and in the dark. However, there are some factors, potentially posttranslational regulation and PEP availability, which reduce *ZmPEPC* activity in vivo.

Keywords C₄ photosynthesis · Leaf ¹³CO₂ discrimination · Leaf dark respiration · *Oryza sativa* · *PEPC* overexpression · Rice

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✉ Asaph B. Cousins
acousins@wsu.edu

¹ School of Biological Sciences, Molecular Plant Sciences, Washington State University, Pullman, WA 99164-4236, USA

² C4 Rice Center, International Rice Research Institute (IRRI), Los Baños, Philippines

³ Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, UK

⁴ Laboratory of Anatomy and Morphology, V.L. Komarov Botanical Institute of the Russian Academy of Sciences, Prof. Popov Street 2, St. Petersburg, Russia 197376

Introduction

Genetic engineering of C₃ plants to perform C₄ photosynthesis will require overexpression of phosphoenolpyruvate carboxylase (PEPC) in the leaf mesophyll (M) cells to catalyze the initial carboxylation reaction (Gehlen et al. 1996;

⁵ Department of Mathematics and Statistics, Washington State University, Pullman, WA 99164-3113, USA

⁶ Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK

⁷ Division of Plant Sciences, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia

Häusler et al. 2002; Fukayama et al. 2003). To date, the over-expression of *PEPC* (*PEPC*-OE) in M cells of several C_3 species (e.g., tobacco, potato, wheat, *Arabidopsis thaliana*, and rice) has shown differences in *PEPC* content, activity, and physiological impacts (Matsuoka et al. 2001; Häusler et al. 2002; Miyao and Fukayama 2003; Zhu et al. 2010; Qi et al. 2017). The presence of transgenic C_4 -type *PEPC* activity in rice enhanced the stomatal and mesophyll CO_2 conductances (g_{sc} and g_m , respectively) and improved leaf net CO_2 assimilation rates (Ku et al. 1999, 2000; Lian et al. 2014). Alternatively, other rice *PEPC*-OE studies showed decreases in leaf net CO_2 assimilation compared to wild-type (WT), particularly under low photorespiratory conditions. This result was suggested to occur because of reduced ribulose-1,5-bisphosphate carboxylase:oxygenase (Rubisco) activity (Fukayama et al. 2003; Taniguchi et al. 2008), and enhanced rates of leaf mitochondrial non-photorespiratory CO_2 release in the light (R_L) (Miyao 2003). Higher R_L in the *PEPC*-OE plants is consistent with the anaplerotic function of *PEPC* (Jeanneau et al. 2002; O'Leary et al. 2011; Abadie and Tcherkez 2018) and a stimulation of the tricarboxylic acid (TCA) cycle with the increased *PEPC* activity (Agarie et al. 2002; Fukayama et al. 2003; Kandoi et al. 2016). In addition, a decrease in O_2 sensitivity in *PEPC*-OE rice plants was reported (Agarie et al. 2002; Fukayama et al. 2003). Collectively, the increase in *PEPC* activity in C_3 plants has provided inconsistent effects on leaf photosynthesis, despite indications that the transferred *PEPC* gene can produce a functional enzyme (Fukayama et al. 2003). In addition, subtle changes in leaf CO_2 assimilation may not be detected with traditional leaf gas exchange measurements.

However, the combined analysis of leaf–atmosphere CO_2 exchange and discrimination against $^{13}CO_2$ can be a useful tool to gain mechanistic insights into changes in leaf carbon metabolism in the light (Farquhar et al. 1989; von Caemmerer 1989; Ghashghaie et al. 2003; Cousins et al. 2007; von Caemmerer et al. 2014). For example, in C_3 plants (instantaneous) leaf net discrimination against $^{13}CO_2$ in the light (Δ_o , ‰) is determined by the discrimination against $^{13}CO_2$ during CO_2 movement from the atmosphere to the chloroplast stroma of M cells, and the $^{13}CO_2$ discriminations associated with carboxylation, photorespiration, and mitochondrial non-photorespiratory respiration (von Caemmerer and Evans 1991; Le Roux et al. 2001; Barbour et al. 2010; Bickford et al. 2010; Tazoe et al. 2011; Evans and von Caemmerer 2013). The $^{13}CO_2$ fractionation due to the carboxylation reactions (b , ‰) includes the contributions of both Rubisco and *PEPC* (Cernusak et al. 2013; Ubierna and Farquhar 2014), where the $^{13}CO_2$ fractionation by Rubisco (b_3) is ~29.0‰ with respect to dissolved CO_2 (Roeske and O'Leary 1984) and is typically assumed to be fairly constant across species and insensitive to temperature (Ghashghaie et al. 2003; Evans and von Caemmerer 2013). By contrast,

the net $^{13}CO_2$ fractionation associated with *PEPC* (b_4) is circa –5.7‰ at 25 °C, which includes the ^{13}C fractionations during CO_2 dissolution in water, catalyzed hydration of CO_2 to bicarbonate (HCO_3^-) and *PEP* carboxylation (von Caemmerer et al. 2014). In C_3 plants, b for gaseous CO_2 ranges between 28.2 and 30‰, corresponding to carboxylation by *PEPC* from 5% of total leaf carbon uptake to zero, respectively (Brugnoli et al. 1988; Gillon and Griffiths 1997; Ghashghaie et al. 2003). When the contribution by *PEPC* activity to total carbon uptake rate is large, for example, in the C_3 – C_4 intermediate *Flaveria floridana* where *PEPC* activity was estimated to be 12 to 20% of the total carboxylation, this can significantly affect Δ_o (Alonso-Cantabrana and von Caemmerer 2016). This impact of *PEPC* activity on Δ_o provides a benchmark for evaluating the contribution of C_4 -*PEPC* (e.g., from *Zea mays*, *ZmPEPC*) activity to carbon uptake in rice.

However, in addition to the carboxylation reactions, photorespiration and mitochondrial non-photorespiratory respiration can also influence Δ_o . For example, Δ_o decreases at a given ratio of intercellular: atmospheric CO_2 partial pressures (C_i/C_a) with the discrimination against $^{13}CO_2$ associated with photorespiration (Δ_p). By contrast, discrimination against $^{13}CO_2$ associated with non-photorespiratory mitochondrial respiration (Δ_r) may decrease or increase Δ_o depending on the offset in $^{13}CO_2$ compositions ($\delta^{13}C$, ‰) between leaf chamber during measurements and growth chamber (Gillon and Griffiths 1997; Ghashghaie et al. 2003; Gong et al. 2015). The absolute $^{13}CO_2$ fractionation due to mitochondrial non-photorespiratory decarboxylating reactions (e , ‰; relative to photosynthetic products) is usually considered to be lower than the $^{13}CO_2$ fractionation of photorespiration (f , ‰; Rooney 1988; Ivlev et al. 1996; Tazoe et al. 2009; Wingate et al. 2007). However, the magnitude of Δ_e (‰) depends on e , the $^{13}CO_2$ signature of the substrates used for respiration (Stutz et al. 2014) and the ratio of R_L to the carboxylation rate. The leaf net discrimination against $^{13}CO_2$ leads to photosynthetic products depleted in the heavier carbon isotope compared to atmospheric CO_2 , and since Δ_o and the carbon pool used by leaf respiration change during the day (Tcherkez et al. 2017a), it is difficult to accurately estimate $\delta^{13}C$ of R_L substrates. In addition, estimates of R_L are indirect, and it has been shown that mitochondrial respiration activity is partially inhibited in the light (Tcherkez et al. 2005). There are also indications that photorespiration plays a role in regulating the degree of R_L inhibition (Abadie et al. 2017; Tcherkez et al. 2017a; Gauthier et al. 2018). Alternatively, rates of leaf CO_2 evolution in the dark (R_d) and $\delta^{13}C$ of dark-respired CO_2 ($\delta^{13}C_{Rd}$, ‰) can be directly measured, where $\delta^{13}C_{Rd}$ largely depends on the $\delta^{13}C$ of substrates feeding respiration, particularly the pool of leaf carbon previously produced in the light (Atkin et al. 1998; Barbour et al. 2007; Lehmann et al. 2016a, b; Gessler

et al. 2017). Higher R_d values in PEPC overexpressing plants have been observed on potato (Gehlen et al. 1996; Häusler et al. 1999; Rademacher et al. 2002) and also rice (Agarie et al. 2002). Therefore, combined analysis of the leaf CO_2 evolution flux in the dark (R_d) and of $\delta^{13}\text{C}_{\text{Rd}}$ may provide useful insights into leaf carbon metabolism in *PEPC*-OE plants versus WT in the light.

The purpose of the current study was to quantify the effects of transgenic expression of *ZmPEPC* on leaf–atmosphere CO_2 exchange and discrimination against $^{13}\text{CO}_2$ in *Oryza sativa*, with particular interest on the net biochemical $^{13}\text{CO}_2$ fractionation due to the carboxylating enzymes (Δ_{bio}). Measurements were made under low photorespiratory conditions (O_2 partial pressure of 1.84 kPa) to minimize the contribution of photorespiration and refixation of photorespired CO_2 to leaf net discrimination against $^{13}\text{CO}_2$. In addition, three atmospheric CO_2 partial pressures ($p\text{CO}_2$) were used (18.4, 35.0, and 92.1 Pa) to potentially manipulate the relative contributions of PEPC and Rubisco to carboxylation. Leaf net biochemical discrimination against $^{13}\text{CO}_2$ ($\Delta_{\text{bio}}\text{‰}$), which includes both Rubisco and PEPC fractionations, was used to estimate the in vivo contribution to carboxylation by PEPC (β) in the *PEPC*-OE plants. Leaf–atmosphere CO_2 and $^{13}\text{CO}_2$ exchanges were also measured in the dark to determine R_d and $\delta^{13}\text{C}_{\text{Rd}}$ to further explain the effect of *ZmPEPC* to leaf carbon metabolism.

Materials and methods

Plant material

Generation of *ZmPEPC*-expressing transgenic rice lines

Generation of transgenic *Oryza sativa* (rice) lines expressing maize (*Zea mays*) *phosphoenolpyruvate carboxylase* (*ZmPEPC*) was done at the International Rice Research Institute (IRRI; Los Baños, Philippines). *Agrobacterium tumefaciens* (strain LBA4404)-mediated transformation was performed following the method described by Hiei and Komari (2006). A pSC0/*ZmPEPC* vector (see Supplementary material, Fig. S1A) containing a full-length genomic fragment (GenBank Accession no. AF234296.1) was created by subcloning *ZmPEPC* from pIG121Hm/*ZmPEPC* (from Mitsue Miyao, National Institute of Agrobiological Sciences, Tsukuba, Japan; Ku et al. 1999) into pSC0 (GenBank, Accession no. KT365905; Lin et al. 2016) using a *Sma*I restriction digest. Expression of *ZmPEPC* is driven by its native promoter and terminator. A co-transformation of pSC0/*ZmPEPC* with pCambia1300, a binary vector with the hygromycin B resistance gene, was performed for selection. Freshly harvested immature embryos of rice (*Oryza sativa* spp. *indica* cv. IR64) 8–12 days after anthesis

were used as explants. After one week of co-cultivation in Murashige and Skoog medium and resting for 5 days, emerging resistant calli were selected with 50 mg L^{-1} of hygromycin B. A total of 83 transgenic rice plantlets regenerated from hygromycin-resistant calli were kept in hydroponics (Yoshida culture solution; Yoshida et al. 1972) for 2 weeks to acclimate. A total of 59 *ZmPEPC* PCR positive plants from these lines were grown in soil. Plants with a single copy of the transgene and more than 50% *ZmPEPC* protein accumulation relative to the maize control were advanced to further generations to obtain homozygous lines. At T_3 generation, homozygosity was confirmed on four plants (events) by DNA blot analysis for T-DNA integration (see Fig. S1B for PEPC-28 event). All four events had abundant *ZmPEPC* accumulation relative to maize as detected by immunoblot (see Fig. S2A). Progeny of the PEPC-28 event showed that detectable *ZmPEPC* localizes to rice M cells (Fig. S2B), and the subsequent T_4 generation (line PEPC-28) was chosen for analysis in the present study. *Oryza sativa* cv. IR64 line A009 was used as a negative control for transgene expression, and *Zea mays* cv. B73 was used as a positive control for protein accumulation throughout.

Plant growth conditions

PEPC-OE (line PEPC-28) and WT plants (cv. IR64, line A009) were grown, together with *Zea mays* cv. B73 plants, in a controlled environment growth chamber (G_{ch} ; Bigfoot series, BioChambers Inc., Winnipeg, MB, Canada) at the School of Biological Sciences at Washington State University, Pullman, WA (USA). Plants were individually grown in 4-L free drainage pots as described in Giuliani et al. (2013). The photoperiod was 14 h, from 8:00 to 22:00 h standard time. Light was provided by F54T5/841HO Fluorescent 4100 K and 40 W Halogen incandescent bulbs (Philips) and was supplied in a bell-shaped pattern during the photoperiod with a maximum photosynthetic photon flux density (PPFD) of $600 \text{ mol photons m}^{-2} \text{ s}^{-1}$ incident on the plant canopy for 10 h. Air temperature was 22°C in the dark, and after switching on the light, it tracked the PPFD pattern with a maximum of 26°C for 10 h. Air relative humidity was maintained at $\sim 70\%$ so that the maximum air Vapor Pressure Deficit (VPD) was $\sim 1.6 \text{ kPa}$. During the photoperiod, the atmospheric $p\text{CO}_2$ in the G_{ch} was enriched with CO_2 supplied by a pressurized tank and maintained at 184.2 Pa ($2000 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$); the $^{13}\text{CO}_2$ composition ($\delta^{13}\text{C}_{G_{\text{ch}}}$) was -41.6‰ ($\pm 0.1 \text{ SE}$; $n = 8$) determined as in Giuliani et al. (2019).

Leaf biochemical analysis

The percentage of PEPC in *PEPC*-OE and WT rice compared to maize was determined by protein immunoblot

technique according to Koteyeva et al. (2015). In the two rice plant types and in maize, the *in vitro* activities of PEPC and Rubisco per unit leaf surface area ($\mu\text{mol m}^{-2} \text{s}^{-1}$) were determined spectrophotometrically as described by Cousins et al. (2007) and Walker et al. (2013), respectively. Leaf malate content ($\text{mmol malate m}^{-2}$) was determined spectrophotometrically based on the method of Hatch (1979) and Edwards et al. (1982). These methods for leaf biochemical analysis are reported in Method S1.

Leaf physiological analysis

System set-up for coupled measurements of leaf-atmosphere CO_2 , H_2O , and $^{13}\text{CO}_2$ exchanges

Measurements were performed in Pullman, WA (mean atmospheric pressure of 92.1 kPa). Two LI-6400XT portable gas analyzers (LI-COR Biosciences, NE, USA; detecting $^{12}\text{CO}_2$) operating as an open system were coupled to a tunable-diode laser absorption spectroscopy, which detects $^{12}\text{CO}_2$ and $^{13}\text{CO}_2$ isotopologs (TDLAS model TGA200A, Campbell Scientific, Inc., Logan, UT, USA). The system set-up was as described in Giuliani et al. (2019), based on Ubierna et al. (2013), Stutz et al. (2014), and Sun et al. (2014).

Leaf photosynthesis was determined with a LI-COR equipped with a $2 \times 3 \text{ cm}^2$ leaf chamber (L_{ch}) and a 6400-02B LED light source (LI-COR Biosciences). Alternatively, leaf dark respiration was determined with an $8 \times 10 \text{ cm}^2$ custom-built L_{ch} having an adaxial glass window, and with a volume of $\sim 100 \text{ cm}^3$ (Barbour et al. 2007, based on Sharkey et al. 1985). The chamber had a hollowed stainless-steel frame sealed with a closed-cell foam gasket and was connected to a circulating water bath for temperature control in the lumen. Before the dark respiration measurements, the leaf portion in the L_{ch} was illuminated with a 6400-18 RGB light source (LI-COR Biosciences).

Protocol for coupled measurements of leaf-atmosphere CO_2 , H_2O , and $^{13}\text{CO}_2$ exchanges

Leaf photosynthetic measurements ($n = 4$ in *PEPC*-OE and $n = 5$ in WT) were taken between 9:00 and 16:00 h standard time; on each plant, the mid-to-distal portions of two fully expanded leaves from the same stem were positioned to completely cover the L_{ch} section. Measurements were taken at atmospheric CO_2 partial pressures in the L_{ch} (C_a) of 18.4, 35.0, and 92.1 Pa (i.e., 200, 380, and 1000 $\mu\text{mol CO}_2 \text{mol}^{-1}$ air, respectively) and with $^{13}\text{CO}_2$ composition entering the L_{ch} (δ_{in} , corresponding to the ^{13}C composition of the CO_2 source from a pressurized tank) of -48.0‰ . The O_2 partial pressure ($p\text{O}_2$) was set at 1.84 kPa (i.e., 20 $\text{mmol O}_2 \text{mol}^{-1}$ air), PPFD was 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, t_{leaf} was 25 °C, and leaf-to-air

VPD was kept in the range of 1.0–1.5 kPa. The airflow rate through the LI-COR system was 300 $\mu\text{mol s}^{-1}$ ($\sim 0.48 \text{ L min}^{-1}$), and gas analyzers were matched after each change in C_a when the TDLAS was not measuring the air leaving the L_{ch} . Leaves were acclimated for about 30 min, and the data were recorded for an additional 30–40 min under each measurement condition. The rate of net CO_2 assimilation per leaf surface area (A , $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$), stomatal conductance to CO_2 diffusion (g_{SC} , $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$), intercellular $p\text{CO}_2$ (C_i , Pa), and the ratio $C_i:C_a$ (C_i/C_a) were determined. The ^{13}C signature of leaf dry matter ($\delta^{13}\text{C}_{\text{dm}}$, ‰) and total N content as fraction (%) of leaf dry matter ($n = 4$ for *PEPC*-OE; $n = 5$ for WT) were determined by isotope ratio mass spectrometry (IRMS) as described in Giuliani et al. (2019). Total N content per unit leaf surface area (g m^{-2}) was then calculated based on leaf dry matter per area.

Leaf dark respiration measurements were performed on two plants per day (one *PEPC*-OE and one WT). Each plant was taken out of the G_{ch} at 9:30 h standard time, and the mid-to-distal portions of 8–9 fully expanded leaves, similar to those used for the photosynthetic analysis, were enclosed in the custom-built L_{ch} to completely cover the section area of $\sim 76 \text{ cm}^2$. Under $p\text{O}_2$ of 1.84 kPa, the leaves were first exposed to 750 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of PPFD for 20 min, 500 for 15 min (at t_{leaf} of 25 °C), and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 5 min (at t_{leaf} of 30 °C). The airflow rate through the LI-COR system was changed from 700, to 500, and to 350 $\mu\text{mol s}^{-1}$ tracking the decreasing PPFD. The measurement CO_2 (supplied by a new cartridge every day) had $\delta^{13}\text{C}$ from -6.2 to -4.8‰ to generate a large $\delta^{13}\text{C}$ difference between the L_{ch} and the G_{ch} atmosphere (-41.6‰). The C_a in the L_{ch} was maintained at 35.0 Pa. The stepwise decrease in PPFD and airflow rate minimized the perturbation to the gas exchange measurements when transitioning the leaf from the light to dark. After 40 min of leaf photosynthesis, dark was imposed in the L_{ch} , and leaf CO_2 evolution was measured at $p\text{O}_2$ of 18.4 kPa and t_{leaf} of 30 °C for 195 min to determine the dynamics of the dark respiration rate per unit (one side) leaf surface area (R_d , $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$) and corresponding $\delta^{13}\text{C}$ ($\delta^{13}\text{C}_{\text{Rd}}$, ‰) ($n = 4$). The t_{leaf} was set at 30 °C to enhance the precision of the dark measurements, and the gas analyzers were matched at the beginning of the dark period and every 15 min thereafter, when the TDLAS was not measuring the leaf chamber air. In addition, three plants ($n = 3$) of the *PEPC*-OE line and of WT were taken out of the G_{ch} at 12:00 h 3 days after their use for photosynthesis measurements and darkened at 25 °C for 24 h. Subsequently, leaf dark CO_2 evolution was measured at t_{leaf} of 30 °C and at $p\text{O}_2$ of 18.4 Pa to determine $R_{d(24\text{h})}$ ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$) and $\delta^{13}\text{C}_{\text{Rd}(24\text{h})}$ (‰). The description of the abbreviations, and symbol and unit of the environmental parameters and leaf variables are listed in Table S1.

The net discrimination against $^{13}\text{CO}_2$ in the light, mesophyll CO_2 conductance, and ^{13}C composition of dark-evolved CO_2

Instantaneous leaf net discrimination against $^{13}\text{CO}_2$ in the light (Δ_o , ‰) was calculated by mass balance according to Evans et al. (1986). The leaf net biochemical discrimination against $^{13}\text{CO}_2$ (Δ_{bio} , ‰), which depends on the biochemistry of net CO_2 uptake, was determined for the *PEPC*-OE plants at the different C_a (using Eq. S1 in Method S2), where the mesophyll conductance to CO_2 diffusion (g_m , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$) is a required input (Alonso-Cantabrana and von Caemmerer 2016). In the applied procedure, Δ_{bio} and g_m are not independent variables), and Δ_{bio} is a proxy of b (the in vivo $^{13}\text{CO}_2$ carboxylation fractionation; see Eq. 1 below) that is necessary to determine g_m . The Δ_{bio} in *PEPC*-OE plants were therefore calculated assuming WT g_m values, which were estimated according to Evans and von Caemmerer (2013) as described in Method S3. Specifically, the mean g_m values determined on the WT plants at C_a of 18.4, 35.0, and 92.1 Pa were used to calculate Δ_{bio} ($n=4$) in *PEPC*-OE plants at the three C_a values. The assumption of equal g_m between *PEPC*-OE and WT plants was supported by the sensitivity analysis of Δ_{bio} on g_m (see Fig. S3) compared with the Δ_{bio} analysis in Alonso-Cantabrana and von Caemmerer (2016) for a C_3 – C_4 intermediate species. In addition, comparable g_m values were determined on the *PEPC*-OE and WT plants from measurements of leaf–atmosphere oxygen (in alternative to carbon) isotope exchange (see Table S2; based on Ubierna et al. 2017; Sonawane and Cousins 2018). However, leaf ^{18}O based g_m is not strictly associated with the biochemistry of photosynthesis as is the ^{13}C based g_m , and therefore, it could not be used in the present analysis to determine Δ_{bio} in *PEPC*-OE plants.

In *PEPC*-OE plants, the fraction of carboxylation by *PEPC* (β , $\text{mol C}_{(\text{by_PEPC})} \text{ mol}^{-1} \text{ C}_{(\text{by_Rubisco+PEPC})}$; $n=4$) was determined by solving for the β value that minimized the difference between the Δ_{bio} determined by eq. S1 and Δ_{bio} modeled ($\Delta_{\text{bio_mod}}$, ‰) based on Griffiths et al. (2007) as

$$\Delta_{\text{bio_mod}} = [b_3 - \beta(b_3 - b_4)] - \frac{R_L e^*}{(A + R_L)} \quad (1)$$

assuming the $^{13}\text{CO}_2$ fractionation of Rubisco (b_3) and *PEPC* (b_4) were 29.0 and -5.7‰ , respectively. The R_L ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) is mitochondrial non-photorespiratory respiration rate in the light; e^* (‰) is the experimental $^{13}\text{CO}_2$ fractionation associated with R_L , and the term $(A + R_L)$ corresponds to the carboxylation rate by Rubisco plus *PEPC*. Under the assumption that at the same t_{leaf} , there was no difference between leaf mitochondrial respiration rate in the light (R_L) and three hours after light–dark transition ($R_{\text{d}(3\text{h})}$, $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$; Table 3), R_L at t_{leaf} of 25 °C was predicted from $R_{\text{d}(3\text{h})}$ at 30 °C using the temperature response

function in Bernacchi et al. (2001). The e^* (-6.4‰) was determined as the difference between $\delta^{13}\text{C}$ of the CO_2 entering the leaf chamber during photosynthetic measurements (-48‰) and in the growth chamber (-41.6‰).

The term $[b_3 - \beta(b_3 - b_4)]$ in Eq. 1 is equivalent to the in vivo $^{13}\text{CO}_2$ carboxylation fractionation (b , ‰), as reported in Farquhar and Richards (1984). Therefore, b can be calculated for the *PEPC*-OE plants using β estimated as described above. Alternatively, $\Delta_{\text{bio_mod}}$ can be predicted in WT assuming b is equal to b_3 (29.0‰), i.e., no b_4 and β values were applied given a negligible in vitro *PEPC* activity in WT plants (see “Results”).

Leaf net discrimination against $^{13}\text{CO}_2$ in the light for *PEPC*-OE and WT plants was predicted based on Ubierna and Farquhar (2014) with a simplified equation as

$$\Delta^{13}\text{C}_{\text{mod}} = a + (b - a) \times C_c / C_a, \quad (2)$$

where $a=4.4\text{‰}$ is the $^{13}\text{CO}_2$ fractionation during CO_2 diffusion through stomata; $b=[b_3 - \beta(b_3 - b_4)]$ for *PEPC*-OE (Eq. 1; see Results) and $b=29\text{‰}$ for WT; C_c is the $p\text{CO}_2$ in the chloroplast (Pa), as calculated by Fick’s first law as $C_c = C_i - A/g_m$.

The ^{13}C composition in leaf respired CO_2 in the dark ($\delta^{13}\text{C}_{\text{Rd}}$, ‰) was calculated according to Barbour et al. (2007), based on Evans et al. (1986).

Given $\delta^{13}\text{C}_{\text{Rd}(i)}$ as the value of $\delta^{13}\text{C}$ for dark-evolved CO_2 at time i (by 3 min over 195 min from light–dark transition), the fractional contribution of L_{ch} carbon assimilates to $\delta^{13}\text{C}_{\text{Rd}(i)}$ ($\delta^{\text{Rd}}_{L_{\text{ch_substr}(i)}} \text{‰} / \text{‰}$) was calculated for *PEPC*-OE and WT plants ($n=4$) according to Giuliani et al. (2019) as

$$\delta^{\text{Rd}}_{L_{\text{ch_substr}(i)}} = \frac{(\delta^{13}\text{C}_{\text{Rd}(i)} - \delta^{13}\text{C}_{\text{Rd}(24\text{h})})}{(\delta^{13}\text{C}_{L_{\text{ch_Ph}}} - \delta^{13}\text{C}_{\text{Rd}(24\text{h})})}, \quad (3)$$

where $\delta^{13}\text{C}_{\text{Rd}(24\text{h})}$ are as given in Table 3, and $\delta^{13}\text{C}_{L_{\text{ch_Ph}}}(\text{‰})$ are the representative $\delta^{13}\text{C}$ of *PEPC*-OE or WT carbon assimilates produced in the L_{ch} before light–dark transition. The assumptions underlying Eq. 3 are described in Giuliani et al. (2019), and the values of the variables used for calculations are presented in Table S3. Based on the total fractional contributions of L_{ch} and G_{ch} assimilates to $\delta^{13}\text{C}_{\text{Rd}}$ equal 1, the complementing fractional contribution of G_{ch} assimilates to $\delta^{13}\text{C}_{\text{Rd}(i)}$ ($\delta^{\text{Rd}}_{G_{\text{ch_substr}(i)}} \text{‰} / \text{‰}$) was determined for both plant types as $\delta^{\text{Rd}}_{G_{\text{ch_substr}(i)}} = 1 - \delta^{\text{Rd}}_{L_{\text{ch_substr}(i)}}$.

Leaf A–C_i response curves

For *PEPC*-OE and WT plants, A–C_i response curves ($n=4$) were determined with the LI-6400XT through stepwise decreases in C_a from 35.0 to 3.7 Pa, at 1.84 kPa $p\text{O}_2$, PPFD of 1500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, t_{leaf} at 25 °C, and VPD between 1.0 and 1.5 kPa. For each response curve, a least

square regression analysis was applied to the initial slope (for $C_i \leq 9.2$ Pa) to calculate the CO_2 compensation point (Γ , Pa).

Statistical analysis

Statistical analyses for the effects of plant-type (*PEPC*-OE and WT) and/or C_a level on the leaf photosynthetic and dark respiration variables are described in Method S4. In addition, a nonlinear model with three parameters was used to fit the R_d and $\delta^{13}\text{C}_{\text{Rd}}$ responses for the two plant types over a 3-hour interval. The significance between the two plant types for the R_d or $\delta^{13}\text{C}_{\text{Rd}}$ responses was inferred from the analysis of the model parameters, i.e., range (difference between the initial value and the lower asymptote), exponential rate of change, and lower asymptote (floor of the response) (see Methods S4 for the description of the procedure).

Results

Leaf biochemical analysis

PEPC content and activity

Mean PEPC content in *PEPC*-OE and WT plants compared to *Z. mays* were $65\% \pm 2.2$ SE and $4.1\% \pm 0.4$ SE, respectively (Fig. 1a; $n=2$). The in vitro mean activities of PEPC in young (expanding) leaves of *PEPC*-OE and WT plants were 52.3 and 4.1 $\mu\text{mol HCO}_3^- \text{m}^{-2} \text{s}^{-1}$, respectively, and 54.9 and 2.2 $\mu\text{mol HCO}_3^- \text{m}^{-2} \text{s}^{-1}$ in mature leaves, respectively. The in vitro mean activity of PEPC in mature *Z. mays* leaves was 280 $\mu\text{mol HCO}_3^- \text{m}^{-2} \text{s}^{-1}$, and thus the PEPC activity in *O. sativa* *PEPC*-OE mature leaves was approximately 25 times greater than WT but ~5 times lower than that in *Z. mays* (Fig. 1b; $n=3$). The mean ratios of in vitro PEPC: Rubisco activity in mature leaves were 0.79 ± 0.07 SE and 0.04 ± 0.01 SE for *PEPC*-OE and WT plants, respectively ($n=3$).

Leaf malate content

The mean malate contents per unit leaf surface area ($\text{mmol malate m}^{-2}$) determined on leaf samples taken immediately after leaf photosynthetic measurements were 0.60 ± 0.13 SE in *PEPC*-OE and 0.55 ± 0.06 SE in WT plants, but not statistically different between plant types ($n=5$; $P>0.05$).

Leaf physiological analysis

Leaf photosynthetic responses

There was a significant $p\text{CO}_2$ effect on A , g_{SC} , C_i , C_i/C_a , C_c , C_c/C_a , and Δ_o , but these parameters did not differ between

Fig. 1 **a** Immunoblot analysis for PEPC from soluble proteins extracted from mature rice leaves, showing protein molecular weight (kDa) and band intensity quantitation. The levels of PEPC for both *PEPC*-OE and WT are mean percentage values of *Z. mays* ($n=2$). **b** In vitro PEPC activity determined in both young and mature leaves of *PEPC*-OE and WT, and mature leaves of *Z. mays* plants. Values are mean $\pm$ SE ($n=3$)

PEPC-OE and WT plants (Table 1). In WT plants, g_m significantly decreased with $p\text{CO}_2$ (Table 2).

A significant plant-type effect was determined on Δ_{bio} (‰), with lower values in *PEPC*-OE plants with respect to WT (Fig. 2a; $P=0.006$), and on b (‰), with lower values in the *PEPC*-OE plants compared to a b of 29.0 in WT plants (Fig. 2a; $P=0.003$). In addition, the fraction of carboxylation by PEPC (β) in the *PEPC*-OE plants was significantly different from β equal to 0 in WT (Fig. 2b; $P<0.001$). The values of Δ_{bio} and b tended to be lower, and β values greater, at C_a of 18.4 Pa compared to the higher $p\text{CO}_2$, but there was not a significant effect of $p\text{CO}_2$ or a plant-type $\times p\text{CO}_2$ effect on these parameters. For *PEPC*-OE and WT plants ($n=4$), the overall means of Δ_{bio} across the three experimental $p\text{CO}_2$ range were 27.1 ± 0.5 SE and 29.2 ± 0.5 SE, respectively. In addition, b overall mean of 26.9 ± 0.5 SE and β overall mean of $\sim 0.06 \text{ mol C}_{(\text{by_PEPC})} \text{mol}^{-1} \text{C}_{(\text{by_Rubisco+PEPC})} \pm 0.01$ SE were calculated for the *PEPC*-OE plants.

The $\Delta^{13}\text{C}_{\text{mod}}$ values for WT and *PEPC*-OE plants significantly fit the corresponding Δ_o values plotted versus C_c/C_a , with slopes of 24.6 ‰ in WT ($R^2=0.93$; $P<0.001$) and 22.5 ‰ ($R^2=0.87$; $P<0.001$) in *PEPC*-OE plants (Fig. 3). The Δ_o plotted versus C_c/C_a was generally higher for the WT compared to *PEPC*-OE plants (Fig. 3), and the regression line fitting Δ_o in the two plant types showed a significantly higher slope in WT (24.8 ‰) compared to *PEPC*-OE

Table 1 Leaf photosynthetic traits of *PEPC*-OE ($n=4$) and WT ($n=5$) plants at different atmospheric C_a and pO_2 of 1.84 kPa

Plant-type	C_a (Pa)	A ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	g_{sc} ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$)	C_i (Pa)	C_i/C_a	C_c (Pa)	C_c/C_a	Δ_o (‰)
<i>PEPC</i> -OE	18.4	14.4 ± 0.5	2.05 ± 0.17	10.3 ± 0.3	0.56 ± 0.02	7.0 ± 0.2	0.38 ± 0.01	12.8 ± 0.4
	35.0	27.0 ± 0.6	2.42 ± 0.14	21.9 ± 0.5	0.62 ± 0.01	15.4 ± 0.5	0.44 ± 0.01	14.7 ± 0.9
	92.1	35.8 ± 1.2	1.89 ± 0.32	68.2 ± 3.0	0.74 ± 0.03	54.1 ± 2.6	0.59 ± 0.03	17.8 ± 1.1
WT	18.4	15.7 ± 0.9	2.32 ± 0.17	10.6 ± 0.3	0.57 ± 0.01	6.9 ± 0.2	0.38 ± 0.01	13.3 ± 0.3
	35.0	26.5 ± 1.9	2.41 ± 0.33	21.6 ± 0.8	0.62 ± 0.02	15.0 ± 1.0	0.43 ± 0.03	14.8 ± 0.7
	92.1	35.2 ± 1.3	1.40 ± 0.09	63.3 ± 1.0	0.69 ± 0.01	49.2 ± 0.6	0.53 ± 0.01	17.4 ± 0.2
Significance	Plant-type	$P=0.953$	$P=0.697$	$P=0.176$	$P=0.460$	$P=0.277$	$P=0.201$	$P=0.914$
	CO ₂ level	$P<0.001$	$P=0.012$	$P<0.001$	$P<0.001$	$P<0.0001$	$P<0.0001$	$P<0.001$
	POC Linear	ns	$P=0.009$	$P<0.001$	$P<0.001$	$P<0.0001$	$P<0.0001$	$P<0.001$
	POC Quadratic	$P<0.001$	ns	ns	ns	ns	ns	ns
	Plant-type $\times$ CO ₂ level	$P=0.708$	$P=0.262$	$P=0.091$	$P=0.149$	$P=0.178$	$P=0.417$	$P=0.788$

A is leaf net CO₂ assimilation rate, g_{sc} is stomatal conductance to CO₂ diffusion, C_i is $p\text{CO}_2$ in the intercellular air space, C_c is chloroplast $p\text{CO}_2$, Δ_o is leaf net discrimination against $^{13}\text{CO}_2$ in the light. In *PEPC*-OE plants, C_c was calculated based on the mesophyll conductance to CO₂ diffusion (g_m) values used for Δ_{bio} analysis, that is, the g_m values determined on WT. Values are mean $\pm$ SE. Significance ($P<0.05$) of the effects of plant-type, CO₂ level, and plant-type $\times$ CO₂ level interaction were evaluated by SAS PROC MIXED. The significance of CO₂ levels was evaluated in terms of linear and quadratic polynomial orthogonal contrasts (POC)

Table 2 Leaf mesophyll CO₂ conductance (g_m) values of WT plants ($n=5$) at different atmospheric C_a , which were applied also to *PEPC*-OE plants for Δ_{bio} analysis

	WT			Significance
C_a (Pa)	18.4	35.0	92.1	
g_m ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$)	4.4 ± 0.4	4.2 ± 0.6	2.6 ± 0.2	$P=0.016$

Values are mean $\pm$ SE. Significance ($P<0.05$) of the effect of CO₂ level was evaluated by SAS PROC MIXED

(23.1‰) plants ($P<0.001$; See Method S4 for statistical analysis).

There was not a significant plant-type effect on $\delta^{13}\text{C}$ of leaf biomass ($\delta^{13}\text{C}_{\text{dm}}$; Table 3); in addition, leaf nitrogen content (g m^{-2}) was not statistically different between *PEPC*-OE and WT plants: 2.0 ± 0.2 SE and 2.5 ± 0.2 SE ($n=4$), respectively. Furthermore, the CO₂ compensation point (Γ , Pa) was not significantly different between transgenic and WT plants, with mean values of 0.70 ± 0.09 SE and 0.61 ± 0.07 SE ($n=4$), respectively.

Leaf dark respiration responses

Before the light–dark transition, the A values at pO_2 of 1.84 kPa and PPFD of $750 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ were similar in the *PEPC*-OE and WT plants, with mean rates of 13.9 ± 0.6 SE and 14.1 ± 0.1 SE $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, respectively ($n=3$). After the light–dark transition, R_d in the *PEPC*-OE and WT plants had a hyperbolic decrease

over the 3-hour interval, with a rapid decline in the first hour (Fig. 4a). The *PEPC*-OE plants showed higher R_d (~25% enhanced rates; see Fig. 4a; Table 3) compared to WT over the three-hour period, with a statistical significance determined based on the analysis of the three-parameter nonlinear model selected to fit the R_d responses. Specifically, a significantly greater R_d lower asymptote was estimated in the *PEPC*-OE versus WT plants ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$; $P<0.0001$), while R_d ranges ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$; $P=1.000$) and R_d exponential rates of change ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ min}^{-1}$; $P=0.114$) did not differ between the two plant types (Table S4). The mean values of R_L inferred from $R_{d(3h)}$ were 0.76 ± 0.05 SE for *PEPC*-OE and 0.56 ± 0.03 SE $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for WT plants.

In the transgenic and WT plants, $\delta^{13}\text{C}_{\text{Rd}}$ after the light–dark transition showed a negative hyperbolic pattern over the three-hour interval, with most of the variations occurring in the first 30 min, and with higher $\delta^{13}\text{C}_{\text{Rd}}$ in the *PEPC*-OE plants compared to WT over the entire

Fig. 2 **a** The net ^{13}C biochemical discrimination (Δ_{bio} ; triangles) and the in vivo $^{13}\text{CO}_2$ carboxylation fractionation (b ; circles) in the *PEPC*-OE (empty symbols) and WT (full symbols) plants at C_a of 18.4, 35.0, and 92.1 Pa. For WT plants $\Delta_{\text{bio}} = \Delta_{\text{bio_mod}}$ and b equal to 29.0‰. **b** The carboxylation by PEPC (β) in *PEPC*-OE plants (empty symbol) compared to β equal to zero in WT (full symbol) at C_a of 18.4, 35.0, and 92.1 Pa. In both plots, symbols correspond to mean value $\pm$ SE (for *PEPC*-OE: $n=3$ at C_a of 35.0 Pa, $n=4$ at C_a of 18.4 and 92.1 Pa; $n=4$ for WT)

dark period (Fig. 4b). The $\delta^{13}\text{C}_{\text{Rd}}$ response in the *PEPC*-OE plants was found to be statistically higher than that in WT plants based on the analysis of the three-parameter nonlinear model selected to fit the $\delta^{13}\text{C}_{\text{Rd}}$ values. In particular, the $\delta^{13}\text{C}_{\text{Rd}}$ ranges were not significantly different (‰; $P=0.157$), but the $\delta^{13}\text{C}_{\text{Rd}}$ floor value was significantly higher (‰; $P=0.003$) in the *PEPC*-OE versus WT plants (Table S5). However, the values of $\delta^{13}\text{C}_{(6\text{min})}$ and $\delta^{13}\text{C}_{(3\text{h})}$ were not significantly different between plant types (Table 3). After 24 h in the dark, there were no significant differences in $R_{\text{d}(24\text{h})}$ and $\delta^{13}\text{C}_{\text{Rd}(24\text{h})}$ between *PEPC*-OE and WT plants.

Fig. 3 Leaf net discrimination against $^{13}\text{CO}_2$ in the light (Δ_o) versus ratio of chloroplast to atmosphere CO_2 partial pressures (C_c/C_a) determined on individual *PEPC*-OE and WT plants ($n=4$) at C_a of 18.4, 35.0, and 92.1 Pa under $p\text{O}_2$ of 1.84 kPa. Closed symbols are for WT, and open symbols are for *PEPC*-OE plants; squares for $C_a=18.4$, circles for $C_a=35.0$ and triangles for $C_a=92.1$ Pa. Lines represent the leaf net discrimination against $^{13}\text{CO}_2$ predicted as $\Delta^{13}\text{C}_{\text{mod}} = a + (b-a) \times C_c/C_a$ (based on Ubierna and Farquhar 2014) where $b=29.0$ ‰ for WT (solid line) and $b=26.9$ ‰ for *PEPC*-OE (dashed line); a equal to 4.4‰

Discussion

Leaf photosynthetic traits in *PEPC*-OE versus WT plants

In the current study, the in vitro leaf activity of PEPC was ~25 times higher in the *PEPC*-OE plants than that in WT, and the mean PEPC activity relative to Rubisco activity was 79% in the transgenic plants compared to 4% in WT. However, the higher in vitro PEPC activity in the *PEPC*-OE plants had no detectable effect on A , or other photosynthetic parameters (e.g., stomatal conductance). Previous studies have also shown no enhancement of A in transgenic rice and tobacco expressing *ZmPEPC*, compared to untransformed plants, even though there was a large increase in in vitro PEPC activity (Taniguchi et al. 2008; Hudspeth et al. 1992). While other studies showed that transgenic rice plants expressing *ZmPEPC* had a lower O_2 sensitivity of A compared to WT, due to a decrease in A at low O_2 level (Ku et al. 1999, 2000; Agarie et al. 2002; Fukayama et al. 2003), other studies showed that under photorespiratory conditions A was higher in transgenic rice expressing *ZmPEPC* (Jiao et al. 2002) or C_4 PEPC from sugarcane (Lian et al. 2014). Moreover, transgenic *Arabidopsis thaliana* expressing *ZmPEPC* with a tenfold increase of in vitro PEPC activity showed ~18% higher A compared to control plants (Kandoi et al. 2016); similar enhancement of A was also seen in

Table 3 Leaf dark respiration rates (R_d , at 30 °C) and ^{13}C composition of R_d after 6 min ($R_{d(6\text{min})}$ and $\delta^{13}\text{C}_{Rd(6\text{min})}$; $n=4$), 3 h ($R_{d(3\text{h})}$ and $\delta^{13}\text{C}_{Rd(3\text{h})}$; $n=4$), and 24 h ($R_{d(24\text{h})}$ and $\delta^{13}\text{C}_{Rd(24\text{h})}$; $n=3$); $n=3$)

Plant-type	$R_{d(6\text{min})}$ ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	$R_{d(3\text{h})}$ ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	$R_{d(24\text{h})}$ ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	$\delta^{13}\text{C}_{Rd(6\text{min})}$ (‰)	$\delta^{13}\text{C}_{Rd(3\text{h})}$ (‰)	$\delta^{13}\text{C}_{Rd(24\text{h})}$ (‰)	$\delta^{13}\text{C}_{dm}$ (‰)
PEPC-OE	1.61 ± 0.10	1.04 ± 0.07	0.74 ± 0.08	-42.7 ± 1.54	-61.6 ± 1.1	-66.1 ± 0.0	-61.8 ± 0.3
WT	1.23 ± 0.04	0.77 ± 0.04	0.69 ± 0.02	-45.2 ± 1.47	-62.4 ± 1.7	-67.2 ± 1.0	-62.3 ± 0.5
Significance	$P=0.014$	$P=0.014$	$P=0.506$	$P=0.304$	$P=0.703$	$P=0.456$	$P=0.473$

In addition, leaf dry matter ^{13}C composition ($\delta^{13}\text{C}_{dm}$; $n=4$ for PEPC-OE; $n=5$ for WT) were determined on transgenic and WT plants. Values are mean $\pm$ SE. Significance ($P < 0.05$) of the effect of plant-type was evaluated by SAS PROC MIXED

Fig. 4 **a** Dynamics of leaf dark respiration rate (R_d) and **b** ^{13}C composition associated with R_d ($\delta^{13}\text{C}_{Rd}$) estimated on PEPC-OE (open circles) and WT (closed circles) after leaf exposure to light at $p\text{O}_2$ of 1.84 kPa. Symbols in **a** and **b** correspond to mean value calculated every three min $\pm$ SE ($n=4$)

determined on PEPC-OE versus WT (grown under atmospheric $\delta^{13}\text{C}$ of -41.6‰) after leaf light exposure at $p\text{O}_2$ of 1.84 kPa

ZmPEPC transgenic wheat (Hu et al. 2012). These different responses to higher C_4 PEPC activities in transgenic plants of rice and other C_3 species highlight the need for further research to clarify the physiological impacts as well as the control of C_4 -PEPC (and in particular *ZmPEPC*) activity in rice leaves.

There are several factors that could limit in vivo C_4 -PEPC activity expressed in a C_3 plant. For example, 3-phosphoglyceric acid (3-PGA) produced by Rubisco could be used for synthesis of PEP to drive additional PEPC reactions to produce oxaloacetate (OAA) and then malate through malate dehydrogenase (MDH). However, the export of PEP from the chloroplast in the cytosol may limit *ZmPEPC* in rice (Taniguchi et al. 2008; Weber and von Caemmerer 2010). Moreover, *ZmPEPC* in rice is known to be in a dephosphorylated status during the light time, and therefore it operates at reduced rates because of the low affinity for PEP and the feedback (allosteric) inhibition by various metabolites such as malate, aspartate, and glutamate (Vidal and Chollet 1997; Jeanneau et al. 2002). This posttranslational PEPC control is involved in mediating carbon–nitrogen interactions; specifically, forms of PEPC with diminished feedback inhibition may increase carbon flux into organic acids (OAA and malate) and amino acids at the expense of starch and soluble sugars (Rademacher et al. 2002; O’Leary et al. 2011). In two previous studies, a 1.5- to 3-fold increase in leaf malate occurred in the light of transgenic tobacco, potato and rice overexpressing PEPC (Hudspeth et al. 1992; Häusler et al. 1999; Rademacher et al. 2002; Ku et al. 2000; Agarie et al. 2002). However, in the current study on rice, the malate content was not significantly higher in PEPC-OE plants compared to WT. The accumulation of leaf malate during the photoperiod in PEPC-OE rice plants will depend on relative rates of malate synthesis via PEPC, rates of catabolism of malate by mitochondria, and rates of export of malate outside the leaves. In general, the net rate of leaf CO_2 assimilation incorporates the CO_2 fluxes through carboxylation, photorespiration, and light respiration, which are difficult to disentangle with traditional measurements of leaf CO_2 exchange. However, as discussed below, the combined

analysis of leaf–atmosphere CO_2 exchange and discrimination against $^{13}\text{CO}_2$ can be a useful tool to gain insights into these various fluxes of CO_2 within the leaf.

Leaf net discrimination against $^{13}\text{CO}_2$ in the light, and net biochemical $^{13}\text{CO}_2$ discrimination in the PEPC-OE plants

In C_3 plants, leaf net discrimination against $^{13}\text{CO}_2$ in the light (Δ_o , ‰) integrates the discrimination against $^{13}\text{CO}_2$ during CO_2 diffusion (in both gas and liquid phases) and due to the carboxylation and decarboxylation reactions. In particular, the Rubisco $^{13}\text{CO}_2$ fractionation (b_3) is 29‰, whereas the PEPC net $^{13}\text{CO}_2$ fractionation associated with bicarbonate fixation (b_4) is -5.7 ‰ (Ubierna and Farquhar 2014; von Caemmerer et al. 2014). Therefore, an increase of the carboxylation by PEPC (β) would lower the in vivo $^{13}\text{CO}_2$ carboxylation fractionation (b) and potentially decrease Δ_o (Farquhar and Richards 1984; Lanigan et al. 2008; Bickford et al. 2010). In the present study, the values of b in the WT were set equal to 29.0‰ at all CO_2 levels, i.e., there was no carboxylation by rice native PEPC given the negligible in vitro PEPC activity determined in WT plants. The leaf net biochemical discrimination against $^{13}\text{CO}_2$ (Δ_{bio}) and b were significantly lower by ~ 2 ‰ in the PEPC-OE compared to WT plants, across the $p\text{CO}_2$ experimental range. Although in PEPC-OE plants there was a tendency for Δ_{bio} to decrease with lower $p\text{CO}_2$ (i.e., β to increase, in accordance with Abadie and Tcherkez 2018), the CO_2 dependency was likely minimized due to the low photorespiratory measurement conditions (Leegood and von Caemmerer 1988, 1989, 1994). However, across the $p\text{CO}_2$ experimental interval the calculated mean β was $\sim 6\%$ in PEPC-OE plants compared to zero in WT (Fig. 2b). The significant change in Δ_{bio} and increase in β indicate a contribution of ZmPEPC to carboxylation in the PEPC-OE plants compared to WT even though there was no detectable difference in A between the plant types. This might suggest either that carbon carboxylated by PEPC did not go through Calvin cycle (Häusler et al. 1999) or that Rubisco had a lower carboxylation efficiency in the PEPC-OE plants compared to WT (Agarie et al. 2002; Fukayama et al. 2003). The latter explanation seems unlikely in the present study, where slightly higher Rubisco activity was determined in the PEPC-OE plants compared to WT, and comparable maximum carboxylation efficiency values ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$) were calculated for the two plant types (data not shown). Alternatively, based on the collective information from previous studies, overexpression of PEPC may have enhanced the anaplerotic pathway (Fukayama et al. 2003; Miyao and Fukayama 2003; O’Leary et al. 2011; Kandoi et al. 2016; Abadie and Tcherkez 2018) rather than promoting carbon fixation of the C_4 -like photosynthetic pathway.

The difference in b between the PEPC-OE and WT plants could in part be affected by the magnitude of $\frac{R_L e^*}{(A + R_L)}$ (that contributes to $\Delta_{\text{bio_mod}}$ in Eq. 1) in the transgenic plants. However, in the current study, the $R_L/(A + R_L)$ ratio was low at all three C_a . In addition, given the relatively low sensitivity of b to R_L (see Fig. S4A), even a higher R_L in the PEPC-OE in comparison to WT plants will have a minor contribution to the ^{13}C discrimination analysis.

In the present study, leaf photosynthetic measurements were conducted under low $p\text{O}_2$ (1.84 kPa) to reduce the uncertain contributions of photorespiration and potential re-fixation of (photo)respired CO_2 by PEPC and Rubisco (Ku et al. 2000; Agarie et al. 2001) to leaf net discrimination against $^{13}\text{CO}_2$, and therefore to minimize the errors in Δ_{bio} estimate. In addition, since low $p\text{O}_2$ reduces the inhibition of R_L relative to rates of leaf respiration in the dark (R_d) (Abadie et al. 2017; Tcherkez et al. 2017a, b; Gauthier et al. 2018), R_L was modelled at 25 °C from R_d at 30 °C after three hours from the light–dark transition. Based on previous studies, there is indication of negative effects exerted by the atmospheric CO_2 level, in the short-term, on leaf respiration activity in the light (Tcherkez et al. 2008, 2017a). Since the predicted R_L were applied over all measurement CO_2 conditions, a potential overestimation of R_L may have therefore risen at the highest C_a ; nevertheless, its effect on the ^{13}C discrimination analysis is considered minor given the low $R_L/(A + R_L)$ ratio. Since there is no evidence in the literature of different down-regulation of R_L in transgenic PEPC-OE compared WT rice the higher R_L predicted for the PEPC-OE compared to WT plants may be due to ZmPEPC activity.

In the Δ_{bio} analysis, the g_m values determined on WT based on leaf net discrimination against $^{13}\text{CO}_2$ were applied to PEPC-OE plants. This assumption of equal g_m in transgenic and WT plants was supported by nonsignificantly different g_m determined by leaf ^{18}O discrimination (Yakir 1998; Gillon and Yakir 2000; Barbour et al. 2016; Ubierna et al. 2017) on the PEPC-OE and WT plants under the same experimental conditions of the present study (Table S2). It is theoretically possible that the increased PEPC activity in the transgenic plants would enhance g_m compared to WT, as previously reported by Alonso-Cantabrana and von Caemmerer (2016) for a C_3 – C_4 intermediate species. However, an increase in g_m would lead to a further decrease in the estimate of Δ_{bio} as a $0.5 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$ raise in g_m lowers Δ_{bio} by ~ 1 ‰ (Fig. S3).

Furthermore, in the present study the Δ_{bio} analysis assumed no $^{13}\text{CO}_2$ respiratory fractionation via TCA cycle (e , ‰; Ghashghaie et al. 2003; Werner and Gessler 2011); however, even a large e would have exerted a minor effect on b and g_m , as presented in Method S5 Fig. S4C and S4D, respectively.

Leaf dark respiration and ^{13}C composition of evolved CO_2 in PEPC-OE versus WT plants

In the present study, following leaf transition from light to dark, R_d , and $\delta^{13}\text{C}_{\text{Rd}}$ showed a hyperbolic decrease over a three-hour period in both plant types, with a significantly higher R_d and $\delta^{13}\text{C}_{\text{Rd}}$ in the PEPC-OE compared to WT plants (Table S4 and Table S5). It has been previously shown that the high R_d in the first 30 min after light–dark transition, *light enhanced dark respiration* (LEDR), results primarily from leaf respiration of substrates as organic acids (in particular malate), produced in the prior light period (Werner and Gessler 2011; Tcherkez et al. 2012, 2015, 2016b; Gessler et al. 2017). Based on the analysis conducted on several species, Lehman et al. (2016b) reported how the leaf respiration rate and ^{13}C composition of evolved CO_2 during the LEDR time may be only weakly related, and how changes in both responses are highly species-specific. In the present study, for both plant types, a close correlation between R_d and $\delta^{13}\text{C}_{\text{Rd}}$ over the three-hour dark period was determined ($r > 0.90$). However, leaf CO_2 evolution in the dark and its $\delta^{13}\text{C}_{\text{Rd}}$ composition contain information about leaf metabolism and respiratory substrates (Lehman et al. 2016b). For example, Barbour et al. (2007) and Gessler et al. (2009) reported for castor bean (*Ricinus communis*) that leaf LEDR mainly comes from the decarboxylation of ^{13}C heavier metabolites, mostly malate, and that the declines in LEDR and $\delta^{13}\text{C}_{\text{Rd}}$ over time are caused by the decrease in malate availability as respiratory substrate. In the dark, leaf malate can be decarboxylated via malic enzyme in mitochondria to make pyruvate, or alternatively, it can be oxidized to OAA via NAD-MDH (Wiskich and Dry 1985; Douce and Neuburger 1987). Pyruvate and OAA can subsequently be used in anaplerotic reactions to replenish TCA cycle intermediates when they are consumed for lipid or amino acid synthesis (Doubnerová and Ryšlavá 2011; Muramatsu et al. 2014; Lehmann et al. 2015, 2016b). In the current study, the R_d integral over 30 min after light–dark transition was $0.48 \text{ mmol CO}_2 \text{ m}^{-2}$ higher in PEPC-OE with respect to WT plants. Theoretically, if the enhancement of LEDR in the PEPC-OE plants during the first 30 min in the dark was due to malate alone, this would have required total $0.12 \text{ mmol malate m}^{-2}$, i.e., ~the double of the leaf malate content determined during the photoperiod in PEPC-OE plants. Since no significant difference in leaf malate accumulation was observed between PEPC-OE and WT plants, other leaf organic acids (e.g., fumarate, citrate; Agarie et al. 2002; Abadie and Tcherkez 2018) produced in the light duration may have also contributed to a bigger pool of dark respiratory substrates in the PEPC-OE compared to WT (Tcherkez et al. 2012; Lehman et al. 2016b). In addition, part of the leaf malate may be in an inactive pool (e.g., in the vacuole) as observed in C_4 plants (Hatch 1979; Arrivault

et al. 2017) and therefore not readily available for LEDR. Another carbon source for R_d in the PEPC-OE plants could be via function of PEPC in the dark, when ZmPEPC has been reported to be in a phosphorylated status (Fukayama et al. 2003; Leegood 2013). In the dark period, by utilizing part of the PEP produced during glycolysis, ZmPEPC in rice could lead to the increased synthesis of malate that can be metabolized in the TCA cycle as substrate for anaplerosis during mitochondrial respiration, with the possibility to raise the CO_2 evolution (Suzuki et al. 2006).

Over the 3 h in the dark, the variations in $\delta^{13}\text{C}_{\text{Rd}}$ for both PEPC-OE and WT plants suggest a decrease in the contribution to R_d of respiratory substrates produced in the leaf chamber (L_{ch}) before the light–dark transition and a complementary increase in the contribution of substrates produced in the growth chamber. The L_{ch} carbon assimilates were estimated to account from ~50% of the substrates for R_d after 6 min to ~30% after 30 min, and only ~10% after three hours in the dark, with no differences between transgenic and WT plants (see Fig. S5). Tcherkez et al. (2010) estimated that recent carbon assimilates in sunflower (*Helianthus annuus*) provided 40–60% of substrates for leaf respiration (via a pool with a half-life of several hours) both in the light and in the dark; similar contribution was determined by Nogués et al. (2004) on French bean (*Phaseolus vulgaris*) for approximately two-hour dark after leaf illumination. The higher $\delta^{13}\text{C}_{\text{Rd}}$ responses in PEPC-OE plants compared to WT during LEDR may partially depend on a relatively greater contribution to leaf dark respiration of organic acids, which are ^{13}C enriched, compared to other respiratory substrates as sugars and amino acids (Lehman et al. 2016b). In particular, during the photoperiod, the PEPC activity in the PEPC-OE plants could promote the production of ^{13}C -enriched OAA, compared to WT, which can be converted to malate by MDH and used to feed leaf respiration after light–dark transition (Barbour et al. 2007; Gessler et al. 2009; Werner et al. 2011; Lehmann et al. 2016b). In addition, the tendency of a higher $\delta^{13}\text{C}_{\text{Rd}(24\text{h})}$ (and $\delta^{13}\text{C}_{\text{dm}}$) in PEPC-OE versus WT plants may indicate that the substrates available for the TCA cycle produced in the G_{ch} had a slightly more enriched ^{13}C composition in the transgenic plants compared to WT. This may suggest that in the growth chamber at current atmospheric $p\text{O}_2$ and $p\text{CO}_2$ of 184 Pa, a possible lower leaf net discrimination against $^{13}\text{CO}_2$ could have occurred in the PEPC-OE plants compared to WT. For both plant types, a lower $\delta^{13}\text{C}_{\text{Rd}(24\text{h})}$ than $\delta^{13}\text{C}_{\text{dm}}$ is in agreement with Tcherkez et al. (2003).

Conclusions

There are uncertainties in the physiological effect of transgenic expression of ZmPEPC in the C_3 plant rice. However, enhancement of PEPC activity is a key step in

engineering C_4 photosynthesis into C_3 plants. In the present study, the transgenic rice plants expressing *ZmPEPC* had higher in vitro PEPC activity, a significant fraction of carbon fixed by PEPC and a decreased Δ_{bio} compared to WT (determined at $p\text{CO}_2$ from below to above current ambient level). However, A was not significantly different between *PEPC*-OE and WT plants, while R_d and ^{13}C composition of leaf dark-evolved CO_2 were higher in the *PEPC*-OE plants *versus* WT, additionally indicating enhanced in vivo PEPC activity in the *PEPC*-OE plants. These results suggest that although *ZmPEPC* appears to be functional in the *PEPC*-OE rice plants, there are some factors likely related to substrate availability (PEP and/or bicarbonate) or posttranslational controls (e.g., involving regulatory phosphorylation) that reduce the activity of the enzyme in vivo during the photoperiod. Insights into these limitations may be discernible with detailed analysis of metabolite pools (organic acids, carbohydrates, and starch), ad hoc estimates of g_m , and magnitude of refixation of photorespired CO_2 compared to WT. This will provide the much needed understanding to further the development of a functioning C_4 photosynthetic cycle in rice.

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Author contributions SK, SC, H-CL, RAC, WPQ, and JMH generated the transgenic plant material; SvC, RG, RTF, GEE, and ABC planned and designed the experiments; RG performed leaf–atmosphere gas and isotope exchange measurements and analyses, and NK performed the biochemical analysis; RG, SvC, RTF, GEE, and ABC interpreted the data; and RG, ABC, and GEE developed and wrote the manuscript.

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interests.

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