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

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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 ^13^CO₂ 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 ^13^CO₂/parenleft.s1/Δbio/parenright.s1 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 ^13^C composition (δ^13^CR_d) 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 ^13^CO₂ discrimination · Leaf dark respiration · *Oryza sativa* · PEPC overexpression · Rice

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;
Collectively, the increase in PEPC activity in C3 plants has been reported (Agarie et al. 2002; Fukayama et al. 2003). The presence of transgenic C4-type PEPC activity in rice enhanced the stomatal and mesophyll CO2 conductances (gs and gm, respectively) and improved leaf net CO2 assimilation rates (Ku et al. 1999, 2000; Lian et al. 2014). Alternatively, other rice PEPC-OE studies showed decreases in leaf net CO2 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 CO2 release in the light (Ri) (Miyao 2003). Higher Ri 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; Kandoli et al. 2016). In addition, a decrease in O2 sensitivity in PEPC-OE rice plants was reported (Agarie et al. 2002; Fukayama et al. 2003). Collectively, the increase in PEPC activity in C3 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 CO2 assimilation may not be detected with traditional leaf gas exchange measurements.

However, the combined analysis of leaf–atmosphere CO2 exchange and discrimination against 13CO2 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; Ghoshghaie et al. 2003; Cousins et al. 2007; von Caemmerer et al. 2014). For example, in C3 plants (instantaneous) leaf net discrimination against 13CO2 in the light (Δo, ‰) is determined by the discrimination against 13CO2 during CO2 movement from the atmosphere to the chloroplast stroma of M cells, and the 13CO2 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 13CO2 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 13CO2 fractionation by Rubisco (bR) is ~29.0‰ with respect to dissolved CO2 (Roeske and O’Leary 1984) and is typically assumed to be fairly constant across species and insensitive to temperature (Ghoshghaie et al. 2003; Evans and von Caemmerer 2013). By contrast, the net 13CO2 fractionation associated with PEPC (bPEPC) is circa −5.7‰ at 25 °C, which includes the 13C fractionations during CO2 dissolution in water, catalyzed hydration of CO2 to bicarbonate (HCO3−) and PEP carboxylation (von Caemmerer et al. 2014). In C3 plants, b for gaseous CO2 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; Ghoshghaie et al. 2003). When the contribution by PEPC activity to total carbon uptake rate is large, for example, in the C4–C3 intermediate Flaveria floridana where PEPC activity was estimated to be 12 to 20% of the total carboxylation, this can significantly affect Δo (Alonso-Cantabrana and von Caemmerer 2016). This impact of PEPC activity on Δo provides a benchmark for evaluating the contribution of C4-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 Δo. For example, Δo decreases at a given ratio of intercellular: atmospheric CO2 partial pressures (Ci/Ca) with the discrimination against 13CO2 associated with photorespiration (Δf). By contrast, discrimination against 13CO2 associated with non-photorespiratory mitochondrial respiration (Δg) may decrease or increase Δo depending on the offset in 13CO2 compositions (δ13C, ‰) between leaf chamber during measurements and growth chamber (Gillon and Griffiths 1997; Ghoshghaie et al. 2003; Gong et al. 2015). The absolute 13CO2 fractionation due to mitochondrial non-photorespiratory decarboxylating reactions (e, ‰; relative to photosynthetic products) is usually considered to be lower than the 13CO2 fractionation of photosynthesis (f, ‰; Rooney 1988; Ivlev et al. 1996; Tazoe et al. 2009; Wingate et al. 2007). However, the magnitude of Δg (‰) depends on e, the 13CO2 signature of the substrates used for respiration (Stutz et al. 2014) and the ratio of Rl to the carboxylation rate. The leaf net discrimination against 13CO2 leads to photosynthetic products depleted in the heavier carbon isotope compared to atmospheric CO2, and since Δo and the carbon pool used by leaf respiration change during the day (Tcherkez et al. 2017a), it is difficult to accurately estimate δ13C of Rl substrates. In addition, estimates of Rl 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 photosynthesis plays a role in regulating the degree of Rl inhibition (Abadie et al. 2017; Tcherkez et al. 2017a; Gautier et al. 2018). Alternatively, rates of leaf CO2 evolution in the dark (Rd) and δ13C of dark-respired CO2 (δ13C, ‰) can be directly measured, where δ13C,‰ largely depends on the δ13C 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 $\delta^{13}$C$_{Rd}$ 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 $^{13}$CO$_2$ in *Oryza sativa*, with particular interest on the net biochemical $^{13}$CO$_2$ fractionation due to the carboxylating enzymes ($\Delta_{bio}$). Measurements were made under low photorespiratory conditions (O$_2$ partial pressure of 1.84 kPa) to minimize the contribution of photorespiration and reflxation of photorespired CO$_2$ to leaf net discrimination against $^{13}$CO$_2$. In addition, three atmospheric CO$_2$ partial pressures ($p$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 $^{13}$CO$_2$ ($\Delta_{bio}$) includes both Rubisco and PEPC frations, was used to estimate the in vivo contribution to carboxylation by PEPC ($\beta$) in the PEPC-OE plants. Leaf–atmosphere CO$_2$ and $^{13}$CO$_2$ exchanges were also measured in the dark to determine $R_d$ and $\delta^{13}$C$_{Rd}$ 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 plG121Hm/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 SmaI 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 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 mol photons m$^{-2}$ 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 ~70% so that the maximum air Vapor Pressure Deficit (VPD) was ~1.6 kPa. During the photoperiod, the atmospheric $p$CO$_2$ in the G$_{ch}$ was enriched with CO$_2$ supplied by a pressurized tank and maintained at 184.2 Pa (2000 µmol CO$_2$ mol$^{-1}$ air); the $^{13}$CO$_2$ composition ($\delta^{13}$C$_{Gch}$) was $-41.6\%$ (±0.1 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 (µmol m⁻² s⁻¹) were determined spectrophotometrically as described by Cousins et al. (2007) and Walker et al. (2013), respectively. Leaf malate content (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 CO₂, H₂O, and ¹³CO₂ 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 ¹²CO₂) operating as an open system were coupled to a leaf dark respiration was determined with an 8 × 10 cm² leaf chamber (COR Biosciences). The system set-up was as described in Giuliani et al. (2019), based on Ubiera et al. (2013), Stutz et al. (2014), and Sun et al. (2014).

Leaf photosynthesis was determined with a LI-COR equipped with a 2 × 3 cm² leaf chamber (Lch) and a 6400-02B LED light source (LI-COR Biosciences). Alternatively, leaf dark respiration was determined with an 8 × 10 cm² custom-built Lch having an adaxial glass window, and with a volume of ~100 cm³ (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 Lch was illuminated with a 6400-18 RGB light source (LI-COR Biosciences).

**Protocol for coupled measurements of leaf–atmosphere CO₂, H₂O, and ¹³CO₂ 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 Lch section. Measurements were taken at atmospheric CO₂ partial pressures in the Lch (Cₐ) of 18.4, 35.0, and 92.1 Pa (i.e., 200, 380, and 1000 µmol CO₂ mol⁻¹ air, respectively) and with ¹³CO₂ composition entering the Lch (δ₁³C, corresponding to the ¹³C composition of the CO₂ source from a pressurized tank) of −48.0‰. The O₂ partial pressure (pO₂) was set at 1.84 kPa (i.e., 20 mmol O₂ mol⁻¹ air), PPFD was 1500 µmol photons m⁻² s⁻¹, tleaf 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 µmol s⁻¹ (~0.48 L min⁻¹), and gas analyzers were matched after each change in Cₐ when the TDLAS was not measuring the air leaving the Lch. 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 CO₂ assimilation per leaf surface area (A, µmol CO₂ m⁻² s⁻¹), stomatal conductance to CO₂ diffusion (gₛC, µmol CO₂ m⁻² s⁻¹ Pa⁻¹), intercellular pCO₂ (Cᵢ, Pa), and the ratio Cᵢ/Cₐ (Cᵢ/Cₐ) were determined. The ¹³C signature of leaf dry matter (δ¹³C 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 Gch 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 Lch to completely cover the section area of ~76 cm². Under pO₂ of 1.84 kPa, the leaves were first exposed to 750 µmol photons m⁻² s⁻¹ of PPFD for 20 min, 500 for 15 min (at tleaf of 25 °C), and 100 µmol photons m⁻² s⁻¹ for 5 min (at tleaf of 30 °C). The airflow rate through the LI-COR system was changed from 700, to 500, and to 350 µmol s⁻¹ tracking the decreasing PPFD. The measurement CO₂ (supplied by a new cartridge every day) had δ¹³C from −6.2 to −4.8‰ to generate a large δ¹³C difference between the Lch and the Gch atmosphere (~41.6‰). The Cₐ in the Lch 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 Lch, and leaf CO₂ evolution was measured at pO₂ of 18.4 kPa and tleaf of 30 °C for 195 min to determine the dynamics of the dark respiration rate per unit (one side) leaf surface area (Rₕ, µmol CO₂ m⁻² s⁻¹) and corresponding δ¹³C (δ¹³Cₕ,‰) (n = 4). The tleaf 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 Gch at 12:00 h 3 days after their use for photosynthesis measurements and darkened at 25 °C for 24 h. Subsequently, leaf dark CO₂ evolution was measured at tleaf of 30 °C and at pO₂ of 18.4 Pa to determine Rₕ(24h) (µmol CO₂ m⁻² s⁻¹) and δ¹³Cₕ(24h),‰. 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 $^{13}$CO$_2$ in the light, mesophyll CO$_2$ conductance, and $^{13}$C composition of dark-voided CO$_2$

Instantaneous leaf net discrimination against $^{13}$CO$_2$ in the light ($\Delta_{b, \%}^{c}$) was calculated by mass balance according to Evans et al. (1986). The leaf net biochemical discrimination against $^{13}$CO$_2$ ($\Delta_{bio, \%}^{c}$), 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 ($g_m$, µmol CO$_2$ m$^{-2}$ s$^{-1}$ Pa$^{-1}$) is a required input (Alonso-Cantarbrana and von Caemmerer 2016). In the applied procedure, $\Delta_{bio}$ and $g_m$ are not independent variables, and $\Delta_{bio}$ is a proxy of $b$ (the in vivo $^{13}$CO$_2$ carboxylation fractionation; see Eq. 1 below) that is necessary to determine $g_m$. The $\Delta_{bio}$ 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 $\Delta_{bio}$ ($n = 4$) 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 $\Delta_{bio}$ on $g_m$ (see Fig. S3) compared with the $\Delta_{bio}$ analysis in Alonso-Cantarbrana 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; Sonnawane 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 $\Delta_{bio}$ in PEPC-OE plants.

In PEPC-OE plants, the fraction of carboxylation by PEPC ($\beta$, mol C$_{by_{PEPC}}$ mol$^{-1}$ C$_{by_{Rubisco+PEPC}}$; $n = 4$) was determined by solving for the $\beta$ value that minimized the difference between the $\Delta_{bio}$ determined by eq. S1 and $\Delta_{bio}$ modeled ($\Delta_{bio_{mod}, \%}^{c}$) based on Griffiths et al. (2007) as

$$
\Delta_{bio_{mod}} = \frac{[b_3 - \beta(b_3 - b_4)] - R_L e^*}{(A + R_L)}
$$

assuming the $^{13}$CO$_2$ fractionation of Rubisco ($b_3$) and PEPC ($b_4$) were 29.0 and $-5.7\%$, respectively. The $R_L$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$) is mitochondrial non-photorespiratory respiration rate in the light; $e^*$ ($\%$) is the experimental $^{13}$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 ($R_{d(3h)}$, µmol CO$_2$ m$^{-2}$ s$^{-1}$; Table 3), $R_L$ at $t_{leaf}$ of 25 °C was predicted from $R_{d(3h)}$ at 30 °C using the temperature response function in Bernacchi et al. (2001). The $e^*$ ($-6.4\%$) was determined as the difference between $\delta^{13}$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 $^{13}$CO$_2$ carboxylation fractionation ($b, \%$), as reported in Farquhar and Richards (1984). Therefore, $b$ can be calculated for the PEPC-OE plants using $\beta$ estimated as described above. Alternatively, $\Delta_{bio_{mod}}$ can be predicted in WT assuming $b$ is equal to $b_3$ (29.0%), i.e., no $b_4$ and $\beta$ values were applied given a negligible in vitro PEPC activity in WT plants (see “Results”).

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

$$
\Delta^{13}C_{mod} = a + (b - a) \times C_f/C_a,
$$

where $a = 4.4\%$ is the $^{13}$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 $b = 29\%$ for WT; $C_f$ is the pCO$_2$ in the chloroplast (Pa), as calculated by Fick’s first law as $C_f = C_i - A/g_m$.

The $^{13}$C composition in leaf respired CO$_2$ in the dark ($\delta^{13}C_{Rd, \%}^{c}$) was calculated according to Barbour et al. (2007), based on Evans et al. (1986).

Given $\delta^{13}C_{Rd(i)}$ as the value of $\delta^{13}$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 $\delta^{13}C_{Rd(i)}(\delta^{13}C_{L_{chsubstr}(i)}, \% / \%)$ was calculated for PEPC-OE and WT plants ($n = 4$) according to Giuliani et al. (2019) as

$$
\delta^{13}C_{L_{chsubstr}(i)} = \frac{\delta^{13}C_{Rd(i)} - \delta^{13}C_{Rd(24h)}}{\delta^{13}C_{L_{ch-Ph}} - \delta^{13}C_{Rd(24h)}}.
$$

where $\delta^{13}C_{Rd(24h)}$ are as given in Table 3, and $\delta^{13}C_{L_{ch-Ph}}$ are the representative $\delta^{13}$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 $\delta^{13}C_{Rd}$ equal 1, the complementing fractional contribution of $G_{ch}$ assimilates to $\delta^{13}C_{Rd(i)}(\delta^{13}G_{chsubstr(i)}, \% / \%)$ was determined for both plant types as $\delta^{13}G_{chsubstr(i)} = 1 - \delta^{13}L_{chsubstr(i)}$.

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 pO$_2$, PPFD of 1500 µmol photons m$^{-2}$ 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 ($I^*$, Pa).

### Statistical analysis

Statistical analyses for the effects of plant-type (PEPC-OE and WT) and/or $C_i$ 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 $\delta^{13}C_{Rd}$ responses for the two plant types over a 3-hour interval. The significance between the two plant types for the $R_d$ or $\delta^{13}C_{Rd}$ 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 $4.1$ µmol HCO$_3^-$ m$^{-2}$ s$^{-1}$, respectively, and $54.9$ and $2.2$ µmol HCO$_3^-$ m$^{-2}$ s$^{-1}$ in mature leaves, respectively. The in vitro mean activity of PEPC in mature Z. mays leaves was $280$ µmol HCO$_3^-$ m$^{-2}$ s$^{-1}$, and thus the PEPC activity in Z. 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 $0.79 \pm 0.07$ SE and $0.04 \pm 0.01$ SE for PEPC-OE and WT plants, respectively ($n=3$).

**Leaf malate content**

The mean malate contents per unit leaf surface area (mmol malate m$^{-2}$) determined on leaf samples taken immediately after leaf photosynthetic measurements were $0.60 \pm 0.13$ SE in PEPC-OE and $0.55 \pm 0.06$ 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 $p$CO$_2$ effect on $A$, $g_s$, $C_i$, $C_i/C_a$, $C_c$, $C_i/C_s$, and $\Delta_o$, but these parameters did not differ between

\[ \text{Table 1: PEPC-OE and WT plants} \]

| Plant-type  | Leaf  | PEPC activity (µmol HCO$_3^-$ m$^{-2}$ s$^{-1}$) |
|-------------|-------|-----------------------------------------------|
| PEPC-OE     | young | $52.3 \pm 5.3$                             |
|             | mature| $54.9 \pm 5.8$                             |
| O. sativa   | young | $4.1 \pm 0.2$                              |
|             | mature| $2.2 \pm 0.4$                              |
| Z. mays cv. B73 | mature| $280.2 \pm 28.5$                           |

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±SE ($n=3$)

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

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

The $\Delta^{13}C_{mod}$ values for WT and PEPC-OE plants significantly fit the corresponding $\Delta_o$ values plotted versus $C_i/C_a$, 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 $\Delta_o$ plotted versus $C_i/C_a$ was generally higher for the WT compared to PEPC-OE plants (Fig. 3), and the regression line fitting $\Delta_o$ 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) | \( A \) (µmol \( CO_2 \) m\(^{-2}\) s\(^{-1}\)) | \( g_{sc} \) (µmol \( CO_2 \) m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\)) | \( C_i \) (Pa) | \( C/C_a \) | \( C_i/C_a \) | \( \Delta_o \) (‰) |
|------------|--------------|---------------|-----------------|--------------|--------|--------|----------|
| PEPC-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 \( P \leq 0.053 \), CO2 level \( P < 0.001 \), POC Linear ns, Quadratic \( P < 0.001 \), Plant-type x CO2 level \( P = 0.708 \), ns.

\( A \) is leaf net \( CO_2 \) assimilation rate, \( g_{sc} \) is stomatal conductance to \( CO_2 \) diffusion, \( C_i \) is \( pCO_2 \) in the intercellular air space, \( C_i \) is chloroplast \( pCO_2 \), \( \Delta_o \) is leaf net discrimination against \( 13CO_2 \) in the light. In PEPC-OE plants, \( C_i \) was calculated based on the mesophyll conductance to \( CO_2 \) diffusion \( (g_m) \) values used for \( \Delta_{bio} \) analysis, that is, the \( g_m \) values determined on WT. Values are mean ± SE. Significance \( (P < 0.05) \) of the effects of plant-type, \( CO_2 \) level, and plant-type x \( CO_2 \) level interaction were evaluated by SAS PROC MIXED. The significance of \( CO_2 \) levels was evaluated in terms of linear and quadratic polynomial orthogonal contrasts (POC).

Table 2  Leaf mesophyll \( CO_2 \) conductance \( (g_m) \) values of WT plants \( (n=5) \) at different atmospheric \( C_a \), which were applied also to PEPC-OE plants for \( \Delta_{bio} \) analysis

| \( C_i \) (Pa) | \( g_m \) (µmol \( CO_2 \) m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\)) |
|---------------|-----------------|
| WT            |                 |
| 18.4          | 4.4 ± 0.4       |
| 35.0          | 4.2 ± 0.6       |
| 92.1          | 2.6 ± 0.2       |

Significance: \( P = 0.016 \).

Values are mean ± SE. Significance \( (P < 0.05) \) of the effect of \( CO_2 \) 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 \( \delta^{13}C \) of leaf biomass \( (\delta^{13}C_{dm}; \) Table 3); in addition, leaf nitrogen content \( (g \text{ m}^{-2}) \) 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 \( CO_2 \) compensation point \( (I_c, \text{ Pa}) \) 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 µmol photons m\(^{-2}\) 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 µmol \( CO_2 \) m\(^{-2}\) 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 \( (Fig. \text{ 4a}) \). 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 \( (\mu mol \text{ CO}_2 \text{ m}^{-2} \text{ s}^{-1}; P < 0.0001) \), while \( R_d \) ranges \( (\mu mol \text{ CO}_2 \text{ m}^{-2} \text{ s}^{-1}; P = 1.000) \) and \( R_d \) exponential rates of change \( (\mu mol \text{ CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ min}^{-1}; P = 0.114) \) did not differ between the two plant types \( (\text{Table S4}) \). 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 \( \mu mol \text{ CO}_2 \text{ m}^{-2} \text{ s}^{-1} \) for WT plants.

In the transgenic and WT plants, \( \delta^{13}C_{Rd} \) 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 \( \delta^{13}C_{Rd} \) in the PEPC-OE plants compared to WT over the entire...
dark period (Fig. 4b). The \( \delta^{13}C_{\text{Rd}} \) 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 \( \delta^{13}C_{\text{Rd}} \) values. In particular, the \( \delta^{13}C_{\text{Rd}} \) ranges were not significantly different (‰; \( P = 0.157 \)), but the \( \delta^{13}C_{\text{Rd}} \) floor value was significantly higher (‰; \( P = 0.003 \)) in the PEPC-OE versus WT plants (Table S5). However, the values of \( \delta^{13}C_{(\text{min})} \) and \( \delta^{13}C_{(3h)} \) were not significantly different between plant types (Table 3). After 24 h in the dark, there were no significant differences in \( R_{\text{d}(24h)} \) and \( \delta^{13}C_{\text{Rd}(24h)} \) between PEPC-OE and WT plants.

**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 \( Zm \)PEPC, 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 \( Zm \)PEPC 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 \( Zm \)PEPC (Jiao et al. 2002) or \( C_4 \) PEPC from sugarcane (Lian et al. 2014). Moreover, transgenic Arabidopsis thaliana expressing \( Zm \)PEPC 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 (Rd, at 30 °C) and 13C composition of Rd after 6 min (Rd6min) and δ13C(Rd6min); n = 4), 3 h (Rd3h) and δ13C(Rd3h); n = 4), and 24 h (Rd24h) and δ13C(Rd24h); n = 3; n = 3) determined on PEPC-OE versus WT (grown under atmospheric δ13C of −41.6‰) after leaf light exposure at pO2 of 1.84 kPa.

| Plant-type | Rd6min (µmol CO2 m⁻² s⁻¹) | Rd3h (µmol CO2 m⁻² s⁻¹) | Rd24h (µmol CO2 m⁻² s⁻¹) | δ13C(Rd6min) (‰) | δ13C(Rd3h) (‰) | δ13C(Rd24h) (‰) | δ13C(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   |

In addition, leaf dry matter 13C composition (δ13Cdm; n = 4 for PEPC-OE; n = 5 for WT) were determined on transgenic and WT plants. Values are mean ± SE. Significance (P < 0.05) of the effect of plant-type was evaluated by SAS PROC MIXED.

ZmPEPC transgenic wheat (Hu et al. 2012). These different responses to higher C4 PEPC activities in transgenic plants of rice and other C3 species highlight the need for further research to clarify the physiological impacts as well as the control of C4-PEPC (and in particular ZmPEPC) activity in rice leaves.

There are several factors that could limit in vivo C4-PEPC activity expressed in a C3 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 CO2 assimilation incorporates the CO2 fluxes through carboxylation, photorespiration, and light respiration, which are difficult to disentangle with traditional measurements of leaf CO2 exchange. However, as discussed below, the combined
analysis of leaf–atmosphere CO₂ exchange and discrimination against ¹³CO₂ can be a useful tool to gain insights into these various fluxes of CO₂ within the leaf.

**Leaf net discrimination against ¹³CO₂ in the light, and net biochemical ¹³CO₂ discrimination in the PEPC-OE plants**

In C₃ plants, leaf net discrimination against ¹³CO₂ in the light (Δₕ, ‰) integrates the discrimination against ¹³CO₂ during CO₂ diffusion (in both gas and liquid phases) and due to the carboxylation and decarboxylation reactions. In particular, the Rubisco ¹³CO₂ fractionation (β) is 29‰, whereas the PEPC net ¹³CO₂ fractionation associated with bicarbonate fixation (b₄) is ~6% (Ubierna et al. 2014). Therefore, an increase of the carboxylation by PEPC (β) would lower the in vivo ¹³CO₂ carboxylation fractionation (b) and potentially decrease Δₕ (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 CO₂ 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 ¹³CO₂ (Δₕbio) and b were significantly lower by ~2% in the PEPC-OE compared to WT plants, across the pCO₂ experimental range. Although in PEPC-OE plants there was a tendency for Δₕbio to decrease with lower pCO₂ (i.e., β to increase, in accordance with Abadie and Tcherkez 2018), the CO₂ dependency was likely minimized due to the low photorespiratory measurement conditions (Leegood and von Caemmerer 1988, 1989, 1994). However, across the pCO₂ experimental interval the calculated mean β was ~6% in PEPC-OE plants compared to zero in WT (Fig. 2b). The significant change in Δₕbio 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 (µmol CO₂ m⁻² s⁻¹ 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 C₄-like photosynthetic pathway.

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

In the present study, leaf photosynthetic measurements were conducted under low pO₂ (1.84 kPa) to reduce the uncertain contributions of photorespiration and potential re-fixation of (photo)respired CO₂ by PEPC and Rubisco (Ku et al. 2000; Agarie et al. 2001) to leaf net discrimination against ¹³CO₂, and therefore to minimize the errors in Δₕbio estimate. In addition, since low pO₂ reduces the inhibition of \( R_c \) relative to rates of leaf respiration in the dark (\( R_d \)) (Abadie et al. 2017; Tcherkez et al. 2017a, b; Gauthier et al. 2018), \( R_d \) 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 CO₂ level, in the short-term, on leaf respiration activity in the light (Tcherkez et al. 2008, 2017a). Since the predicted \( R_d \) were applied over all measurement CO₂ conditions, a potential overestimation of \( R_d \) may have therefore risen at the highest \( C_a \); nevertheless, its effect on the ¹³C discrimination analysis is considered minor given the low \( R_d/(A+R_d) \) ratio. Since there is no evidence in the literature of different down-regulation of \( R_d \) in transgenic PEPC-OE compared WT rice the higher \( R_d \) predicted for the PEPC-OE compared to WT plants may be due to ZmPEPC activity.

In the Δₕbio analysis, the \( g_m \) values determined on WT based on leaf net discrimination against ¹³CO₂ 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 ¹⁸O 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 C₃–C₄ intermediate species. However, an increase in \( g_m \) would lead to a further decrease in the estimate of Δₕbio as a 0.5 µmol CO₂ m⁻² s⁻¹ Pa⁻¹ raise in \( g_m \) lowers Δₕbio by ~1‰ (Fig. S3).

Furthermore, in the present study the Δₕbio analysis assumed no ¹³CO₂ 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 Figs. 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 $\delta^{13}$C$_{Rd}$ showed a hyperbolic decrease over a three-hour period in both plant types, with a significantly higher $R_d$ and $\delta^{13}$C$_{Rd}$ 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 $\delta^{13}$C$_{Rd}$ over the three-hour dark period was determined ($r$ > 0.90). However, leaf CO$_2$ evolution in the dark and its $\delta^{13}$C$_{Rd}$ 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 $\delta^{13}$C$_{Rd}$ 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 mmol CO$_2$ 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 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 $\delta^{13}$C$_{Rd}$ 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 Noguès et al. (2004) on French bean (Phaseolus vulgaris) for approximately two-hour dark after leaf illumination. The higher $\delta^{13}$C$_{Rd}$ 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 $\delta^{13}$C$_{Rd(24h)}$ (and $\delta^{13}$C$_{dm}$) 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 $p$O$_2$ and $p$CO$_2$ of 184 Pa, a possible lower leaf net discrimination against $^{13}$CO$_2$ could have occurred in the PEPC-OE plants compared to WT. For both plant types, a lower $\delta^{13}$C$_{Rd(24h)}$ than $\delta^{13}$C$_{dm}$ 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 C₄ photosynthesis into C₃ 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 Δbio compared to WT (determined at pCO₂ from below to above current ambient level). However, A was not significantly different between PEPC-OE and WT plants, while Rg and %C composition of leaf dark-evolved CO₂ 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 gₘₐₚ and magnitude of refixation of photorespired CO₂ compared to WT. This will provide the much needed understanding to further the development of a functioning C₄ 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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