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Overexpression of C4 PEPC caused O_2-insensitive photosynthesis in transgenic rice plants

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Abstract

We have introduced an intact maize gene for phospho*enol*pyruvate carboxylase (PEPC) into C3 plants, rice (*Oryza sativa* cv. Kitaake). Most transgenic rice plants showed high-level expression of the maize gene. PEPC was two to three times more active in the leaves of some transgenic plants than in maize leaves. In transformants, the sensitivity of photosynthesis to O_2 inhibition was reduced with the increased activity of the maize C4 PEPC. However, the alleviation of O_2 inhibition was not due to an increase in the partial direct fixation of atmospheric CO_2 via the enhanced maize PEPC, but rather due to the reduced stimulation of photosynthesis with a subatmospheric O_2 level. Pi feeding to the leaves restored CO_2 assimilation rate under the subatmospheric O_2 condition, and consequently, the O_2 inhibition in the transformants increased to a level comparable to that of the non-transformants. These results suggested that the O_2-insensitive photosynthesis in the PEPC transformants was caused by a Pi limitation of photosynthesis. The activities of two key enzymes for sucrose synthesis, SPS and FBPase, and the sucrose and starch content were reduced in the leaves of the transformants. On the other hand, the dark respiration rate and the malate content in the leaves increased in the transformants. These results indicated that enhanced PEPC activity led to a decrease in the availability of Pi in chloroplast via a reduction of the activities of the key enzymes responsible for Pi recycling and it also caused an increased consumption of the substrate (triose-phosphate) in respiration, but not in sucrose biosynthesis.

Keywords: C3; C4; O_2 insensitivity; PEPC; Pi limitation; Transgenic rice

1. Introduction

In the leaves of C4 plants, phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) catalyzes the initial fixation of atmospheric CO_2 in the photosynthesis pathway. PEPC fixes CO_2 (as HCO_3^-) into the C4 acid oxaloacetic acid, which is rapidly converted to malate and/or aspartate. The C4 acid is transported to the gas-tight bundle sheath cells (BSC), where decarboxylation takes place. CO_2 is released for fixation by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), as is a C3 product, pyruvate, which is used for the regeneration of the HCO_3^- acceptor, PEP [1]. The C4 photosynthetic pathway acts as a 'CO_2 pump' to raise the CO_2 concentration in BSC at the site of Rubisco. C3 plants, in which Rubisco serves as a primary enzyme for CO_2 fixation, suffer from O_2 inhibition of photosynthesis due to the oxygenase activity of Rubisco and the subsequent loss of CO_2 through photorespiration. A high CO_2 partial pressure in the BSCs in C4 plants suppresses the oxygenase activity of Rubisco and enables it to operate near its maximum catalytic rate. In turn, this trait confers a number of advantages on C4

Abbreviations: A, CO_2 assimilation rate; FBPase, fructose 1,6-bisphosphatase; MDH, malate dehydrogenase; NADP-ME, NADP-malic enzyme; PCK, phospho*enol*pyruvate carboxykinase; PEPC, phospho*enol*pyruvate carboxylase; PPDK, pyruvate orthophosphate dikinase; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; SPS, sucrose phosphate synthase.

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plants, such as enhanced photosynthetic capacity, a high growth rate, and high nutrient and water use efficiencies.

Recently, several attempts have been made to transfer features of the C4 photosynthetic pathway to C3 plants using genetic engineering techniques [2–11]. The cDNA of the gene for PEPC from various sources has been introduced into tobacco [2,3] and potatoes [4,10], the gene for pyruvate orthophosphate dikinase (PPDK) has been introduced into *Arabidopsis* [5] and potatoes [6], and the cDNAs for NADP-malic enzyme (NADP-ME) [9,11] and phospho*enol*pyruvate carboxykinase (PCK) [8] have been introduced into rice. However, the activities of C4 photosynthetic enzymes in transgenic C3 plants were much lower than in C4 plants, and no significant impact on photosynthetic characteristics was observed in most cases. A transgenic potato that overexpressed PEPC from *Corynebacterium glutamicum* had a lowered CO_2 compensation point, with increased rates of respiration both in darkness and in light [12]. Lipka et al. [7] reported that a transgenic potato expressing the NADP-ME of *Flaveria pringlei* in chloroplasts and the PEPC of *C. glutamicum* in cytosol reduced the electron requirements for CO_2 assimilation under high light intensities and at high temperatures. More recently, Suzuki et al. [8] reported that transgenic rice that expressed C4-type PCK exhibited significant incorporation of radioactivity into C4 compounds in labeling experiments with $^{14}CO_2$, but showed no enhancement of the photosynthetic CO_2 assimilation rate.

We have previously demonstrated that the introduction of a maize intact gene for C4-specific PEPC from maize (*Zea mays*) into rice led to an extremely high-level expression of the enzyme in the transgenic rice plants [13]. The sensitivity of the transformants to the inhibition of photosynthesis by O_2 decreased with increasing PEPC activity; atmospheric O_2 inhibited photosynthesis in the non-transgenic plants by 30% while reducing it by about 20% in the transgenic plants. Therefore, we concluded that the reduction of O_2 inhibition in transgenic plants resulted in part from the direct fixation of atmospheric CO_2 by the maize PEPC. However, further analyses suggested that this was not the case. O_2 inhibition of photosynthesis is assessed on the basis of the percentage increase in the CO_2 assimilation rate that is stimulated by subatmospheric levels of O_2 in C3 plants. However, the calculated values of O_2 inhibition are also reduced when CO_2 assimilation did not increase under 2% O_2. We show here that the observed suppression of O_2 inhibition of photosynthesis by means of the overexpression of PEPC resulted from O_2-insensitive photosynthesis, which would likely be caused by Pi limitation of photosynthesis.

2. Materials and methods

2.1. Plant transformation and culture

The maize PEPC gene used was an 8.8-kb fragment containing all exons, introns, and promoter (1.2 kb) and terminator (2.5 kb) sequences [14]. The gene was cloned into pIG121Hm and introduced into calli derived from rice (cv. Kitaake) using *Agrobacterium*-mediated transformation [15]. Transgenic plants were regenerated from hygromycin-resistant calli, planted in soil, and grown in a naturally illuminated greenhouse. The day/night growth temperatures were 27/22 °C, and at the midday peak PPFD was approximately 2000 μmol photons m^{-2} s^{-1}. The plants were fertilized twice with commercial fertilizers before the experiments began. Newly expanded leaves from 6 to 8-week-old-plants were used for all experiments.

2.2. Enzyme extraction and assays

Leaf tissue was harvested from the mid-portion of nine to ten newly mature leaves at around noon (about 6 h after sunrise) on a sunny day, and was immediately immersed in liquid nitrogen and stored at −80 °C temporally until analyzed. The frozen tissue (0.1 g) was homogenized using a mortar and pestle in 1.5 ml of extraction buffer containing 50 mM Tris–HCl (pH 7.0), 10 mM $MgCl_2$, 1mM EDTA, 5 mM dithiothretol, 5% insoluble polyvinylpolypyrrolidone (PVP) and 10% glycerol. For assaying PPDK activity, the extraction buffer was supplemented with 2 mM pyruvate and 2.5 mM phosphate. After total maceration, an aliquot was taken for chlorophyll determination, and the rest of the crude extrant was centrifuged at 15 000*g* for 10 min. The supernatant was used immediately for the assays of Rubisco, PEPC, malate dehydrogenase (MDH), NADP-ME, and PPDK. PEPC and PPDK activities were determined by the method of Slack and Hatch [16] and Aoyagi and Bassham [17], respectively. The activities of NADP-ME, Rubisco, and MDH were assayed according to Hatch and Mau [18], Du et al. [19], and Johnson and Hatch [20], respectively. All enzyme assays were carried out at 30 °C with a spectrophotometer (UV1600, Shimadzu, Kyoto, Japan). For assaying sucrose phosphate synthase (SPS) and fructose 1,6-bisphosphatase (FBPase), the frozen leaf pieces (about 3 cm^2) were extracted in 2 ml of extraction buffer with 0.1 g sea sand and 20 mg of PVP. The brei was centrifuged at 15 000*g* for 10 min. The supernatant was desalted by passing it through a 5 ml Sephadex G-25 column (Hitrap Desalting, Pharmacia Biotech, Sweden). The filtrate was used immediately for the assays of SPS and cytosolic FBPase. SPS was assayed by measuring F6P dependent formation of sucrose-phosphate (+sucrose) from UDPglucose [21]. The assay mixture (70 μl) con-

tained 50 mM HEPES–NaOH (pH 7.5), 15 mM $MgCl_2$, 3 mM UDP glucose, 4 mM F-6-P, 20 mM glucose-6-phosphate (G-6-P), and an aliquot of leaf extract. Mixtures were incubated at 30 °C for 10 min, and reactions were terminated by the addition of 70 μl of 1.0 N NaOH. The resulting mixtures were then kept in a Heat Block at 100 °C for 10 min to destroy unreacted F-6-P and fructose. After cooling, 0.25 ml of 0.1% (v/v) of resorcinol in 95% ethanol and 0.75 ml of 30% HCl were added, and the tubes were incubated at 80 °C for 8 min. Absorbance at 520 nm was measured with the spectrophotometer, and sucrose concentrations were estimated with respect to a standard curve constructed from sucrose standards. FBPase was assayed according to Kerr et al. [21]. The activities of all enzymes were calculated on a protein or chlorophyll basis. The concentration of total soluble protein was determined using Bio-Rad (USA) protein assay reagent. Chlorophyll content was measured by the method of Arnon [22].

2.3. Extract and determination of metabolites

The leaves were harvested as described in the enzyme assay. The metabolites were extracted according to the methods of Leegood and Furbank [23] and Usuda [24]. About 10 cm^2 of leaf pieces were pulverized in liquid N_2 in a pre-cooled pestle and mortar; 1.0 ml of frozen 3% $HClO_4$ was added to the powder and gently pulverized with it. The mortar and pestle were rinsed twice with 0.5 ml ice-cold 3% $HClO_4$. The combined mixture was left for 30 min on ice and centrifuged at 3000*g* for 10 min at 4 °C. The supernatant was neutralized to pH 6.0 with 5 M K_2CO_3 and centrifuged at 10 000*g* for 5 min at 4 °C. The supernatant was immediately used for the determination of metabolites. Metabolite concentrations were determined spectrophotometrically based on the estimated production or consumption of NADPH measured at 340 nm with the spectrophotometer. Malate was measured according to the method of Du et al. [25]. Pyruvate and PEP were measured in a two-step system as described by Du et al. [25]. Aspartate and citrate were measured according to the methods of Bergmeyer et al. [26] and Mollering [27], respectively.

2.4. Carbohydrate analysis

The frozen tissue (3 cm^2), which was an adjacent portion used for the enzyme assay, was homogenized using a mortar and pestle in 1.0 ml of extraction buffer containing 20 mM HEPES–NaOH (pH 7.4). After total maceration, the crude extrant was incubated at 100 °C for 2 min and centrifuged at 4 °C for 10 min. The supernatant was used for sucrose determination. Sucrose was determined with a sucrose content kit (F-kit, Boehringer-Mannheim, Germany) according to the manufacturer's instructions. Particulates including starch were collected by centrifugation and extracted with 80% ethanol until the tissue was pigment free. The pellet was suspended in 20 mM HEPES–NaOH (pH 7.4) and incubated at 100 °C for 2 h. Starch hydrolysis and glucose determination were carried out using the starch determination kit of Boehringer-Manheim (F-kit, Germany).

2.5. Contents of total carbon and nitrogen

The adjacent portion used for the enzyme assay was dried at 80 °C for at least 24 h. The dried samples were ground using a mortar and pestle. The contents of total carbon and nitrogen in 30 mg of the ground sample were measured with an N–C analyzer (Sumigraph model NC-8, Sumitomo Chemical, Japan) and a gas chromatograph (Shimadzu, GC-8A, Japan) using 10 mg hippuric acid as a standard.

2.6. Steady-state measurements of the CO_2 assimilation rate

The steady-state photosynthetic CO_2 assimilation rate (A) was measured in an open mode at 30 °C, 1200 $\mu mol\,m^{-2}\,s^{-1}$, 360 $\mu l\,l^{-1}$ CO_2 and 2 (A_2) or 21% O_2 (A_{21}) according to the method of Hirasawa et al. [28]. The middle sections (10 cm^2) of two or three newly expanded leaves from each plant were sealed in the leaf cuvette. The percent of O_2 inhibition of photosynthesis was calculated as $(A_2 - A_{21}) \times 100/A_2$ [13].

2.7. Time course measurements of the CO_2 assimilation rate and dark respiration rate

The time course of the CO_2 assimilation rate (A) was measured on the uppermost fully expanded leaves of the main culm and the primary tillers using a system for gas exchange measurement with an infrared gas analyzer (model LI-6400, LI-COR, USA). The middle sections of the leaves (3 cm^2) were kept for 30 min in the assimilation chamber, maintained at 1600 $\mu mol^{-2}\,s^{-1}$ of light, 25 °C of leaf temperature, 1.0 kPa of vapor pressure differences (VPD) and 350 $\mu l\,l^{-1}$ of CO_2. Leaves were detached by cutting their leaf sheaths at 15 cm below the auricle in distilled water and were kept in the water for 5 min. The light was turned off and the leaves were kept in the dark for 5 min. After the values reached a constant level, dark respiration rate was measured. The light was turned on and the leaves were kept in the same conditions described above until the A reached a constant level (for 20–30 min). The O_2 concentration [O_2] rapidly changed from 21 to 2%, and A was measured every 10 s for 35 min. At the end of the measurements, [O_2] returned to 21%, and the leaf was kept under the same conditions as described above

(1600 μmol^{-2} s^{-1} of light, 25 °C of leaf temperature, 1.0 kPa of VPD and 350 μl l^{-1} of CO_2) for 10 min. The distilled water was changed to 10 mM KH_2PO_4, and the leaf sheath of the detached leaf was kept in the phosphate solution for 10 min. The Pi solution was then changed to distilled water, and the leaves were kept in the dark for 5 min to equilibrate their metabolic status. The stable values were measured as dark respiration rate of leaves with applied Pi. The light was turned on, and the leaf was kept in 1600 μmol^{-2} s^{-1} of light, 25 °C of leaf temperature, 1.0 kPa of VPD and 350 μl l^{-1} of CO_2 for about 20 min until A become constant. A was monitored every 10 s for 35 min under the different O_2 levels.

3. Results

3.1. Expression of maize PEPC in the transgenic rice plants

Our previous studies demonstrated that the promoters of the maize genes encoding C4-specific PEPC can drive high-level expression of reporter genes in transgenic C3 plants [29,30]. Based on these results, we have introduced intact maize genes into rice, cv. Kitaake. The maize PEPC gene used was an 8.8-kb fragment containing all exons, introns, and promoter (1.2 kb) and terminator (2.5 kb) sequences [14]. As expected, the introduction of the intact gene was effective in increasing the PEPC activity of the rice plants (Fig. 1). A wide range of PEPC activity was detected among the leaves of the individual primary transformants. The majority (85%) of the transgenic plants exhibited PEPC activity that was two to 30-fold that observed in the non-transformants, and approximately 10% of the transgenic rice plants exhibited PEPC activity ranging from 40 to 120-fold that of non-transgenic rice, which corresponds to 1–3 times the level found in maize leaves. All transgenic plants showed normal morphology and fertility, and all produced seeds, suggesting that high levels of expression of the transgene did not interfere with the growth and fertility of most of the transgenic plants.

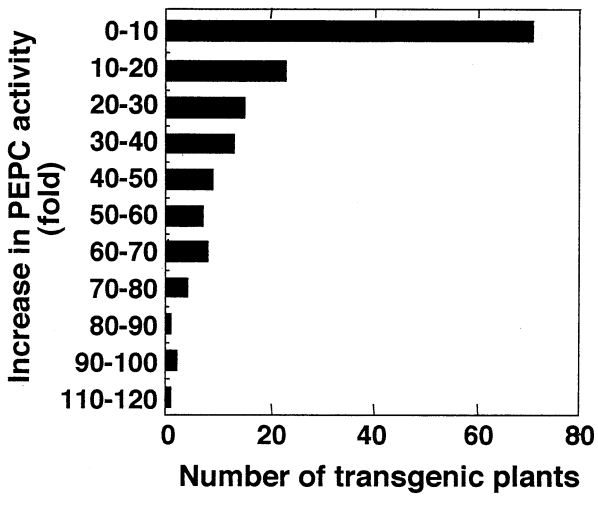

Fig. 1. Frequency distribution of the PEPC activities of leaves of the primary (T1) transgenic rice plants. The PEPC activities ranged from 0.003 to 2.7 μmol mg protein^{-1} min^{-1} in the transgenic rice plants. The enzyme activities, determined on the protein basis, were expressed as fold increased relative to that of non-transgenic rice (0.015 μmol mg protein^{-1} min^{-1}). The PEPC activity of maize was around 40-fold (0.63 μmol mg protein^{-1} min^{-1}) over that of non-transgenic rice.

3.2. Steady-state photosynthesis under 2% O_2

The C3 plant suffers from O_2 inhibition of photosynthesis because of the oxygenase activity of the bifunctional enzyme, Rubisco. In contrast, C4 plants show a negligible level of O_2 sensitivity due to a high CO_2 affinity of PEPC, and their CO_2 concentrating mechanisms in BSCs help to reduce the oxygenase activity of Rubisco. To examine the effect of high-level expression of PEPC on O_2 inhibition of photosynthesis in transgenic rice plants, we measured the steady-state CO_2 fixation rate (A) under two different O_2 levels in non-transformants and transformants (Fig. 2). We chose nine lines of T2 generation, which express PEPC activities ranging from approximately ten to 100-fold higher than those expressed in non-transformants.

The O_2 inhibition of photosynthesis, assessed from the percentage of stimulation observed as O_2 was reduced, decreased progressively with increasing PEPC activity in the transgenic plants (Fig. 2B). However, this reduction was caused by a decrease in the stimulation of photosynthesis that occurred with the decreasing O_2 levels (Fig. 2A). Under 2% O_2, A decreased with the increase of PEPC activity, showing a significant relationship between PEPC activity and O_2 inhibition ($r = 0.90$), whereas A was almost unchanged independent of PEPC activities under 21% O_2. Therefore, this O_2 inhibition resulted from O_2-insensitive photosynthesis rather than from increased CO_2 uptake fixed by PEPC under 21% O_2.

3.3. Activities of C4-related and sucrose synthesis related enzymes

For further analysis, we chose a transformant line of T3 generation designated as PE2-6. Table 1 shows the activities of enzymes of this line contained PEPC, the other C4-related enzymes and sucrose synthesis related enzymes. The PEPC activity of PE2-6 was 1.7-fold that of maize (40 times that of non-transformants). There was the effect of increased PEPC activity on the activities of MDH and Rubisco in the transformants; MDH

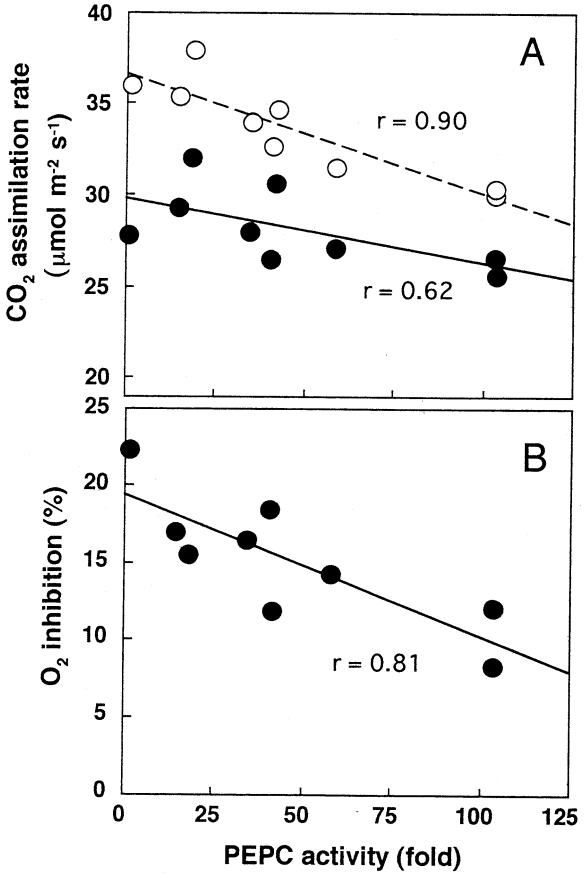

Fig. 2. Photosynthetic CO_2 assimilation rates (A) and oxygen inhibition of photosynthesis (B) as a function of PEPC activity in transgenic rice plants carrying the intact maize PEPC gene. CO_2 assimilation rates were measured under 21 (●) and 2% (○) oxygen (A). The lines and *r* are the regression lines and regression coefficient of the CO_2 assimilation rate (A) and the O_2 inhibition (B) on inverse in PEPC activity.

increased to 1.5-fold activity that of non-transformants, while Rubisco was decreased up to 85% of the level in the non-transformants. SPS and FBPase were reduced to 70 and 40% of the levels in the non-transformants, respectively.

Table 1
The activities of C4-related enzymes and sucrose-synthesis-related enzymes in leaves of maize, the transgenic- and the non-transgenic rice plants

Enzyme	Maize	Non-transformant (μmol mg Chl^{-1} h^{-1})	PE2-6
PEPC	977.2 ± 26.1^b	38.6 ± 2.2^c	1627.4 ± 19.7^a
MDH	1102.7 ± 35.3^a	33.8 ± 1.5^b	52.0 ± 6.3^b
NADP-ME	297.0 ± 16.9^a	14.6 ± 0.4^b	13.3 ± 0.5^b
PPDK	139.5 ± 8.2^a	22.9 ± 1.0^b	21.7 ± 1.3^b
Rubisco	175.9 ± 7.1^c	597.7 ± 33.0^a	511.0 ± 9.1^b
SPS	ND	46.0 ± 1.3^a	32.6 ± 0.6^b
FBPase	ND	44.8 ± 3.2^a	17.4 ± 2.2^b

Data are means ± SE of the three replicates. Means denoted by the same letter did not differ significantly at $P<0.05$ based on Duncan's multiple range test. ND, not determined.

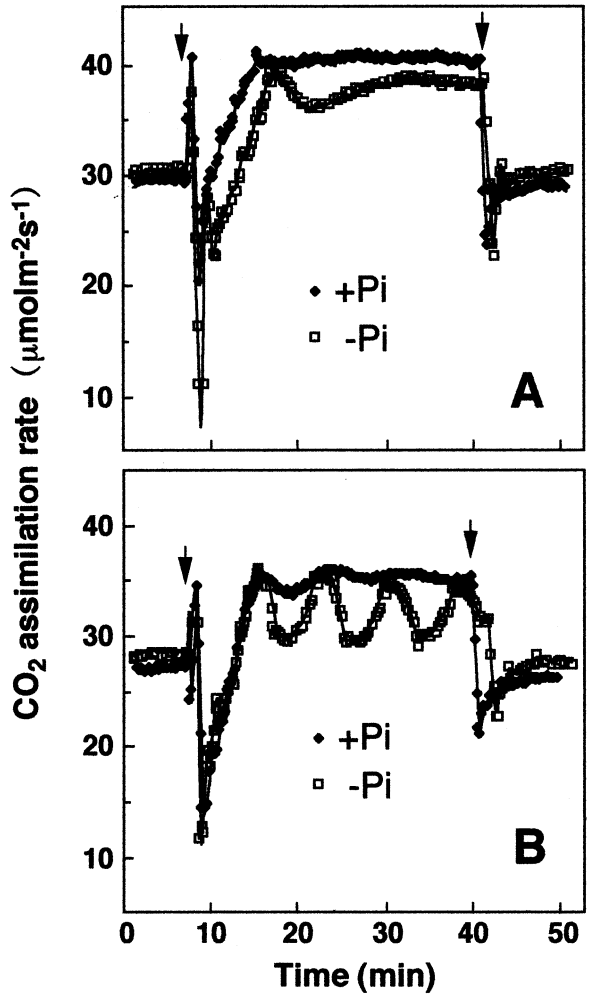

Fig. 3. Effects of phosphate feeding on the CO_2 assimilation rate in the non-transgenic (A) and transgenic (B) rice plants during transitions from 21 to 2% O_2, CO_2 was 350 μl l^{-1} and leaf temperature was 26 °C. The gas phase was switched from 21 to 2% O_2 at around 10 min and 2–21% at around 40 min after the start of the measurements (arrows). Light intensity was 1600 μmol m^{-2} s^{-1}. Note that strong oscillations of CO_2 assimilation were observed in the leaf of transformants (PE2-6), which has 1.7-fold PEPC activities that of maize. The peaks occurred approximately at 8-min intervals. The oscillation disappeared by the phosphate feeding.

3.4. Oscillated photosynthesis under 2% O_2 observed in the transformants

To analyze the O_2 insensitivity of leaf photosynthesis in the PEPC transgenic rice plants, we measured the time course of *A* following the reduction of O_2 levels in the PE2-6 and non-transformants (Fig. 3). Under atmospheric O_2 levels (21%), there were no significant differences in *A* between the leaves of the non-transformants and the PE2-6, showing 29.1 ± 1.0 and 30.9 ± 0.9 μmol m^{-2} s^{-1}, respectively. However, the kinetics of *A*

Table 2
Effect of phosphate feeding on oxygen inhibition of CO_2 assimilation rate and dark respiration rate in the transgenic and the non-transgenic rice plants

	Pi	CO_2 assimilation rate ($\mu mol\ m^{-2}\ s^{-1}$)		O_2 inhibition (%)	Dark respiration rate ($\mu mol\ m^{-2}\ s^{-1}$)
		21% O_2	2% O_2		
Non-transformant	−	30.9 ± 0.9^a	39.2 ± 1.1^a	21.3 ± 0.2^b	0.98 ± 0.09^a
	+	29.2 ± 1.9^a	40.7 ± 1.8^a	28.4 ± 1.4^a	1.01 ± 0.14^a
PE2-6	−	29.1 ± 1.0^a	34.2 ± 1.2^b	14.9 ± 0.8^c	1.46 ± 0.10^b
	+	28.1 ± 0.6^a	36.5 ± 1.0^{ab}	23.1 ± 0.3^b	1.52 ± 0.09^b

The O_2 inhibition was calculated from the values obtained in the measurements shown in Fig. 3. In the transformants the average values were calculated from the maximal and minimum values in the oscillation of the CO_2 assimilation rate. Note that the phosphate feeding led to an increase of the O_2 inhibition of CO_2 assimilation of the transformants to the levels comparable to that of the non-transformants. Data were expressed as means ± SE of the three replicates. Means denoted by the same letter did not differ significantly at $P<0.05$ based on Duncan's multiple range test.

in response to the O_2 reduction were completely different between PE2-6 and the non-transformants. Upon reduction of the O_2 levels from 21 to 2%, *A* of PE2-6 oscillated, ranging from approximately 30 to 36 $\mu mol\ m^{-2}\ s^{-1}$ over about 8 min cycles. In contrast, in the non-transformants *A* decreased to 90% of its maximum value at around 22 min after the start of the measurements, and it recovered gradually and reached steady-state levels at around 40 min without oscillation. Table 2 shows the average values of the three independent gas-exchange measurements in the transformants and non-transformants. The average *A* was decreased in PE2-6 under 2% O_2, although *A* was almost the same between the transformants and non-transformants. Consequently, O_2 inhibition was significantly reduced in the transformants. The reduction of O_2 inhibition was consistent with the results of T2 generation (Fig. 2), suggesting that this characteristic has been conserved hereditarily in the transgenic rice plants.

It has been suggested that the occurrence of O_2-insensitive photosynthesis is caused by the deprivation of phosphate (Pi) for photosynthesis (Pi limitation) [31]. Experimentally, the oscillation and O_2-insensitive photosynthesis were eradicated by phosphate feeding [32] while mannose feeding to the leaf disks increased their frequencies [33]. To test whether the oscillation and O_2 insensitiveness in the transformants were caused by Pi limitation, we fed 10 mM Pi to detached leaves in their transpiration streams for 10 min (see Section 2), and we measured the time course of *A* under two different O_2 levels. In PE2-6, the Pi feeding led to an increase of *A*, and the oscillation in CO_2 assimilation disappeared. As a consequence, the O_2 inhibition of PE2-6 increased to levels comparable to that of the non-transformants (Table 2). These results indicate that the reduced O_2 inhibition in transgenic rice plants could be due to a decrease of CO_2 fixation in 2% O_2, which was caused by Pi limitation of photosynthesis.

3.5. Dark respiration rate

The dark respiration rate increased in the transformants to 1.5-fold that of the non-transformants. The increased dark respiration rate in PEPC transgenic C3 plants was consistent with the results from transgenic potatoes [4]. However, Pi feeding has no effect on the dark respiration rate in the leaves of either the transformants or the non-transformants (Table 2).

3.6. Contents of carbon metabolism intermediates

Table 3 shows the level of respiration-related intermediates and the sugar content in the PE2-6 and the non-transformants. The levels of pyruvate and phospho*enol*pyruvate were decreased in the transformants to approximately 30% of the levels in the non-transformants. In the transformants, malate accumulated to three-fold the amount in the non-transformants, whereas sucrose and starch were decreased to 87 and 97% of the levels in the non-transformants, respectively.

Table 3
Levels of metabolites in the transgenic and the non-transgenic rice plants

Metabolite	Non-transformant	PE2-6
	nmol mg Chl^{-1}	
Pyruvate	737.4 ± 205.6	213.6 ± 51.3
PEP	773.5 ± 219.9	253.3 ± 23.3
Aspartate	269.4 ± 24.2*	181.7 ± 12.5
Citrate	50.8 ± 14.2	54.3 ± 14.1
Malate	75.8 ± 11.3	227.9 ± 30.2*
	μmol mg Chl^{-1}	
Sucrose	44.4 ± 3.2	38.8 ± 8.9
Starch	9.6 ± 2.2	9.0 ± 2.4

Data are means ± SE of the three replicates. *, statistically significant at the 5% level based on Student's *t*-test.

Table 4
Contents of total carbon and nitrogen in the non-transgenic and the transgenic rice plants

	Total carbon (%)	Total nitrogen (%)	Ratio (C/N)
Non-transformant	40.2 ± 0.9	3.4 ± 0.2*	11.8 ± 0.7*
PE2-6	41.0 ± 0.3	4.3 ± 0.1	9.5 ± 0.2

Data are means ± SE of the three replicates. *, statistically significant at the 5% level based on Student's *t*-test.

3.7. Contents of total carbon and nitrogen

Table 4 shows the total carbon and nitrogen contents of the PE2-6 and the non-transformants. There were no significant differences in carbon content between them, but the nitrogen content of the PE2-6 was significantly increased. As a result, the ratio of carbon to nitrogen (C/N) was decreased in the PE2-6.

4. Discussion

In the present study we confirmed our previous results that the introduction of intact maize genes encoding PEPC gives rise to high-level expression of the corresponding C4 enzymes in a C3 plant, rice [13]. This result supports our previous conclusion that C3 plants possess the necessary genetic machinery to express C4-specific genes at high levels [29,30]. The high-level expression of the C4 gene has been inherited in the transgenic rice plants over three generations without interfering with growth and fertility. The results indicated that this is a successful strategy for installing the key biochemical component of the C4 pathway of photosynthesis in C3 plants.

The high-level expression of maize PEPC in transgenic rice plants results in considerable changes in the photosynthetic characteristics in the transformants; the sensitivity of photosynthesis to O_2 inhibition was reduced in a PEPC-activity-dependent manner (Figs. 2 and 3 and Table 2). This result was consistent with our previous results, in which we suggested that this reduction of O_2 inhibition was due to an increase in the partial direct fixation of atmospheric CO_2 via the enhanced maize PEPC [13]. In the present study, however, the reduction of O_2 inhibition in the transformants was due to decreased CO_2 fixation in lowered O_2 (2%), not to an increase of CO_2 fixation via the PEPC under an atmospheric O_2 level (21%) (Fig. 2 and Table 2). Pi feeding to the detached leaves led to an increase of A under the lowered O_2 levels, and to a decrease of the occurrence of the oscillation of A that was observed only in transformants under 2% O_2. Consequently, the O_2 inhibition in the transformants was restored to levels comparable to that of non-transgenic plants (Table 2). Therefore, we suggest here that reduction of A under lowered O_2 levels by overexpression of PEPC could be caused by the deprivation of phosphate (Pi) for photosynthesis in chloroplasts (i.e. Pi limitation) [31,32,34].

The Pi limitation of photosynthesis is considered to be mainly caused by a limitation of triose-P utilization and its concomitant effects of reducing the production of ATP needed for ribulose-1,5-bisphosphate regeneration in the turnover of the photosynthetic reduction (PCR) cycle [31]. The triose-P generated via the PCR cycle is converted to sucrose in the cytosol. In this process, Pi is released and is then available to allow further export of triose-P from the chloroplast. Some Pi will be released within the storoma via starch synthesis, but this process is usually slower (by a factor of three to four) than maximal CO_2 fixation, whereas the release of Pi in sucrose synthesis via the reaction of a phosphatase occurs as quickly as the plant can synthesize triose-P. Therefore, the process of sucrose synthesis is believed to contribute mainly to the Pi recycling. In the present study, the activities of key enzymes for sucrose synthesis, SPS and FBPase (Table 1), and the sucrose and starch content (Table 3) were reduced in the leaves of the PEPC transformants. These results indicate that the amounts of Pi released from those synthesis reactions were diminished by the overexpression of PEPC.

It has been suggested that SPS can be regulated by (i) allosteric control with G-6-P acting as an activator and Pi as an inhibitor [35,36], and (ii) reversible protein phosphorylation with SPS-protein phosphatase (dephosphorylation/activation) and SPS-kinase (phosphorylation/inactivation). Also, Huber and Kaiser [37] have postulated that an increase in certain amino acids (especially Glu and Gly) could inhibit SPS-PP and thereby promote phosphorylation and the inhibition of SPS. The enhanced PEPC may be involved in the reduction of SPS activity by facilitating the production of these amino acids.

It is likely that the reduction of FBPase could be caused by allosteric control with a regulatory metabolite of FBPase, Fru-2,6-P2. The inhibition of SPS would result in increased concentrations of its substrates, in particular Fru-6-P, and in turn, the rise in Fru-6-P would result in an increased concentration of Fru-6-P2 [38]. Further investigation of the contents of such metabolites and the activities of those enzymes in the PEPC transgenic rice plants that exhibit various PEPC activities would clarify the roles of PEPC in the regulatory process for SPS and FBPase.

Triose-P is used both for sucrose biosynthesis and to provide glycolytic PEP for the PEPC-catalyzed formation of oxaloacetate (OAA). We have therefore also

assumed that the reduction of Pi release was caused by an increased consumption of the substrate (triose-P) in respiration. In the transformants, CO_2 evolution through respiration (dark respiration) and malate content were 1.5 and three-fold the values in non-transformants, respectively (Table 2). Similar phenomena were observed in transgenic potatoes, showing that respiration rates increased with increased PEPC activity [4,12]. These results suggest that the overexpression of PEPC likely enhanced the flux of photoassimilates into glycolysis and subsequently into the citric acid cycle [39].

It has been considered that PEPC in C3 plants intrinsically functions in the anaplerotic pathway to replenish the organic acid biosynthesis pathway with C4 chains when demand for amino acid biosynthesis is high; PEPC is also considered to be involved in the refixation of CO_2 evolved via respiration [40,41]. Therefore, it is expected that enhanced PEPC activity in leaves results in a decrease of respiratory CO_2 evolution or even in a recapture of CO_2 evolved via photorespiration. However, if the produced malate through reaction catalyzed by PEPC is decarboxylated by mitochondrial NAD malic enzyme and the resultant pyruvate is completely oxidized in the citric acid cycle, a maximum of four CO_2 would be released for each HCO_3^- fixed by PEPC. Only one HCO_3^- fixed by PEPC is released directly in the form of CO_2 via NAD malic enzyme, whereas the other three CO_2 molecules are derived from glycolytically produced PEP (i.e. due to the action of pyruvate dehydrogenase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase). Thus, as the rates of PEP carboxylation are increased, more CO_2 is probably released than HCO_3^- fixed [39].

The overexpression of PEPC resulted in a decrease of the C/N ratio due to an increased nitrogen content in the transformant (Table 4). This result supported the idea that the enhanced PEPC activity facilitates the feeding of carbon into the TCA cycle and the provision of precursors for amino acid biosynthesis.

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