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High-level expression of maize phospho*enol*pyruvate carboxylase in transgenic rice plants

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Using an *Agrobacterium*-mediated transformation system, we have introduced the intact gene of maize phospho*enol*pyruvate carboxylase (PEPC), which catalyzes the initial fixation of atmospheric CO_2 in C_4 plants into the C_3 crop rice. Most transgenic rice plants showed high-level expression of the maize gene; the activities of PEPC in leaves of some transgenic plants were two- to threefold higher than those in maize, and the enzyme accounted for up to 12% of the total leaf soluble protein. RNA gel blot and Southern blot analyses showed that the level of expression of the maize PEPC in transgenic rice plants correlated with the amount of transcript and the copy number of the inserted maize gene. Physiologically, the transgenic plants exhibited reduced O_2 inhibition of photosynthesis and photosynthetic rates comparable to those of untransformed plants. The results demonstrate a successful strategy for installing the key biochemical component of the C_4 pathway of photosynthesis in C_3 plants.

Keywords: transformation, rice, maize, C_4 photosynthesis pathway, phospho*enol*pyruvate carboxylase

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One of the challenges facing plant biotechnologists is to modify photosynthesis so as to achieve increases in net carbon gain. More than 95% of the terrestrial plants, including many agronomically important crop species, such as rice and wheat, assimilate carbon through the C_3 pathway of photosynthesis[1]. However, C_3 photosynthesis suffers from O_2 inhibition due to the oxygenase reaction of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the subsequent loss of CO_2 from photorespiration[2]. Under current atmospheric conditions (21% O_2, 0.035% CO_2), O_2 reduces the photosynthetic efficiency by as much as 40%. This reduction in photosynthetic efficiency could increase further under drought and high temperature conditions by decreased concentration of CO_2 in the leaf due to closure of stomata. Although there was some interest in engineering Rubisco in favor of carboxylase over oxygenase, current chemical evidence suggests it is unlikely that Rubisco can be engineered for increased carboxylation efficiency[3].

With some modification in leaf anatomy, C_4 plants, such as maize and many weedy species, have evolved a biochemical mechanism to overcome O_2 inhibition of photosynthesis. The C_4 photosynthetic pathway serves as a "CO_2 pump" to concentrate atmospheric CO_2 at the site of Rubisco and thus suppresses its oxygenase activity and the associated photorespiration[4,5]. In the C_4 pathway, atmospheric CO_2 is initially fixed into the C_4 acid, oxaloacetating, in the cytosol of mesophyll cells via phospho*enol*pyruvate carboxylase (PEPC). PEPC has a high affinity for the substrate, bicarbonate, and is not inhibited by O_2. Because of the CO_2 concentration mechanism, photosynthesis by C_4 plants is near saturated at atmospheric CO_2 levels and exhibits little O_2 inhibition and photorespiration[6,7]. Thus, C_4 plants have a selective advantage (high photosynthetic capacity, and high water and nutrient use efficiency) over C_3 plants, especially under low CO_2 conditions in which carbon loss from O_2 inhibition and photorespiration becomes maximal[8].

Evaluation of the potential to transfer C_4 traits into C_3 plants to improve their photosynthetic characteristics has been an active research area in plant biology[6,9]. In addition, enzymes involved in C_4 photosynthesis may also play important roles in plant defense responses to biotic and abiotic stress. For example, upregulation of nicotinamide-adenine dinucleotide phosphate (NADP)-malic enzyme by wounding, low oxygen, low temperature, salinity, and ultraviolet irradiation has been reported in both C_3 (rice[10], bean[11,12]) and C_4 (maize[13]) plants. Metabolic alterations in response to stress allow plants to adapt to adverse conditions. Thus, overexpression of some C_4 enzymes in C_3 plants could confer enhanced tolerance under stress conditions. Conventional hybridization between C_3 and C_4 plants has been successful only in a few plant genera and none of them are crop species[9]. Furthermore, most of the hybrids exhibit infertility due to irregular chromosome pairing or genetic barriers. Thus, employing traditional breeding methods to incorporate C_4 traits into C_3 crops seems difficult. With the development of plant genetic engineering, transfer of foreign genes into crops has become increasingly routine, providing a new approach to altering plant traits. Some attempts have been made in the past to enhance the activities of C_4 photosynthesis enzymes in dicotyledonous C_3 plants (tobacco, potato) through recombinant DNA techniques[14–17]. However, the activities of the C_4 enzymes (e.g., PEPC, NADP-malate dehydrogenase) in transgenic plants were low, and consequently, no significant impact on the physiology of photosynthesis was observed. The introduced genes used in these studies mainly consisted of cDNA clones encoding the C_4 enzymes from monocotyledonous C_4 plants (maize, sorghum) and regulatory sequences, such as the 35S promoter of the cauliflower mosaic virus and nopaline synthetase terminator, for expression in C_3 plants.

We have introduced an intact maize gene for one of the key enzymes in C_4 photosynthesis, PEPC, into rice, a very important

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C_3 crop. High levels of expression of the transgene were obtained in transgenic plants, which did not interfere with growth and fertility of most of the transgenic plants. More importantly, transgenic rice plants with high levels of expression of the maize C_4 enzyme exhibited reduced sensitivity of photosynthesis to O_2 inhibition.

Results

Transformation. Using an *Agrobacterium tumefaciens*-mediated gene transfer system[18], we have transformed two rice cultivars, *Kitaake* and *Nipponbare*, with an intact maize gene encoding C_4-specific PEPC (Fig. 1). A total of 89 primary transgenic rice plants (T1 plants) were obtained from antibiotic-resistant cells: 58 from *Kitaake* and 31 from *Nipponbare*. Only about 10% of the transgenic plants showed abnormal morphology, such as stunted growth, reduced plant height, variegated albino leaves, narrow leaves, or infertility. All other plants exhibited normal phenotype with a usual life cycle, grew to maturity, flowered, and set seeds. Thus, only a small fraction of the transgenic plants have shown genetic damage. This result is similar to others reported earlier with *Agrobacterium*-based transgenic rice plants expressing various promoter-reporter fusion genes[19–21].

Transgenic rice plants expressed high levels of maize PEPC. The level of expression of the maize C_4-specific *pepc* gene in transgenic rice plants was determined first by assaying the activity of PEPC in leaf protein extract, followed by gel electrophoresis and western blot analyses. A wide range of PEPC activity was detected among the transgenic plants; most of them had high levels of activity (Fig. 2). Only three transgenic plants of *Kitaake* had PEPC activities lower than those in untransformed plant. For both cultivars, the majority (85%) of the transgenic plants had activities 2- to 30-fold higher than untransformed plants, with the remaining 15% showing 30- to 110-fold higher activity, or 1- to 3-fold the maize PEPC activity. The specific activity of PEPC in maize leaves (2.25 µmol/mg protein/min) is about 35-fold higher than that in rice leaves (0.06 µmol/mg protein/min). A similar result was obtained with the transgenic plants derived from *Nipponbare*. Furthermore, the high-level expression of the maize PEPC was inherited by the next generations (T2 and T3) in a Mendelian manner, and the enzyme remained active (data not shown). The results demonstrate that the maize gene is stably integrated into the rice genome and expressed normally in the progenies.

Electrophoretic analysis of leaf protein revealed the presence of a novel 106 kDa polypeptide, similar to that of maize PEPC, in the transgenic rice plants (Fig. 3A). Furthermore, among the transgenic plants, the amounts of the 106 kDa polypeptide, as judged from band intensity, correlated well with the enzyme activities. Consistent with this, western blot analysis unequivocally demonstrated the correlation between the amount of PEPC polypeptide and the activity of PEPC among the transgenic rice plants (Fig. 3B). Kinetic analyses also showed that, similar to maize PEPC, the enzyme isolated from transgenic rice plants has a higher K_M for phospho*enol*pyruvate (2 mM) than the enzyme isolated from untransformed rice plants (0.2 mM). These results suggest that the elevated PEPC activities and protein amounts in the transgenic rice plants are due to the expression of the maize enzyme and that the maize enzyme remains functional in rice plants. Based on densitometric determination, the amounts of PEPC in the transgenic rice plants expressing high levels of PEPC were estimated to be 10–12% of the total leaf soluble protein (e.g., 725-43 and 718-3, Fig. 3A). Earlier attempts to increase C_4-specific PEPC activity in transgenic C_3 plants reported only 0.5- to 3-fold increases in activity and small increases in protein amount relative to untransformed plants, which were detectable only by immunological assay, not by protein staining[14–16].

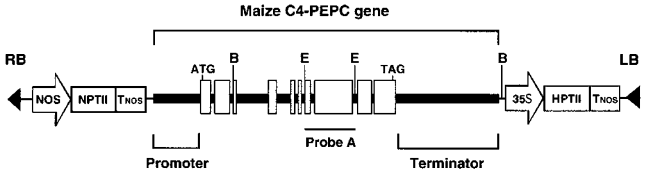

Figure 1. Schematic representation of the intact maize *pepc* gene and the selective antibiotic resistance gene (hygromycin phosphotransferase, HPT II) used for rice transformation. The maize *pepc* gene is an 8.8 kb fragment containing all exons, introns, and the promoter (from –1212) and the terminator (about 2.5 kb) sequences[37]. Probe A used for the Southern hybridization analysis is an *Eco*RI fragment 1.0 kb in size, which is indicated by a thin line. E and B indicate *Eco*RI and *Bam*HI sites, respectively.

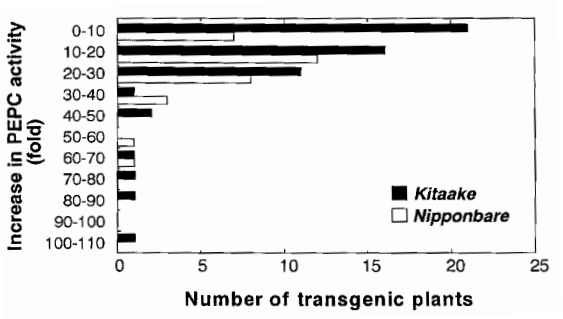

Figure 2. The activities of PEPC in primary transgenic rice plants. PEPC activities ranged from 0.04 to 6.76 µmol/mg/min in transgenic rice plants. The enzyme activity was assayed at room temperature as described previously[38] and expressed as fold increases relative to those in untransformed plants of *Kitaake* and *Nipponbare* (0.06 µmol/mg protein/min). *Kitaake* (solid column); *Nipponbare* (hatched column). Note that PEPC activity of maize leaves is about 35-fold higher (2.25 µmol/mg protein/min) than that of untransformed rice plants when assayed under the same conditions.

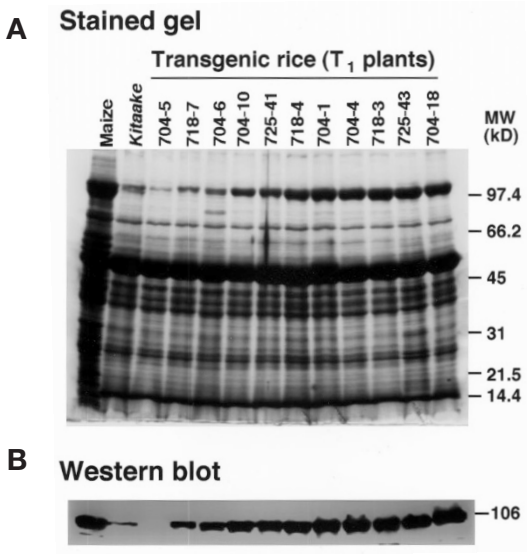

Figure 3. (A) Composition of leaf soluble protein extracted from maize (50 µg), *Kitaake*, and primary (T1) transgenic rice plants derived from *Kitaake* (35 µg). Protein was stained with Coomassie brilliant blue R-250. (B) Western immunodetection of PEPC in leaf protein of maize, *Kitaake*, and transgenic rice plants derived from *Kitaake* using antimaize PEPC serum as probe (17.5 µg protein per lane). The PEPC activities in the leaf extracts of maize and *Kitaake* were 2.25 and 0.06 µmol/min/mg protein, respectively. Samples for the transgenic plants of *Kitaake* were arranged from left to right with increasing PEPC activity (from 0.04 to 3.86 µmol/min/mg protein). Note that the two major proteins in maize leaf, PEPC (106 kDa) and pyruvate, orthophosphate dikinase (95 kDa), cannot be resolved by sodium dodecyl sulfate gel due to similarity in molecular mass.

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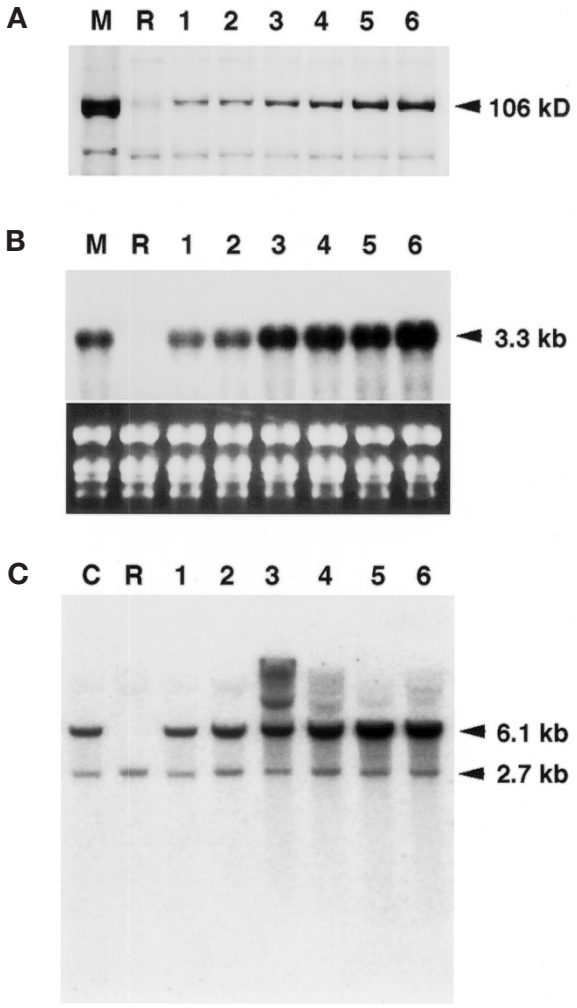

Figure 4. (A) Composition of leaf soluble protein, (B) RNA gel blot, and (C) Southern blot analyses of six independent transgenic rice plants (lane 1–6) with maize (lane M) and untransformed rice (lane R). Six primary transgenic plants with various levels of PEPC activity ranging from 0.23 (lane 1) to 2.27 μmol/min/mg protein (lane 6) were used for these experiments. (A) The intensity of the PEPC polypeptide on SDS-PAGE correlated well with the enzyme activity. (B) Total leaf RNAs (5 μg) from the six transformants, nontransgenic rice and maize, were electrophoresed, transferred onto nitrocellulose membrane, and hybridized with a *Bam*HI fragment of the maize cDNA. A strong 3.3 kb band that comigrated with the *pepc* mRNA in maize (lane M) was detected in the six transformants with increasing intensity, whereas no band was seen in the nontransformant (lane R). Bands of RNAs stained with ethidium bromide in the same gel are shown in the lower panel. (C) For Southern blot analysis, 5 μg genomic DNA from these plants were used. The rice genomic DNA from nontransformant was electrophoresed on lane R. Plasmid DNA (0.13 ng) corresponding to one copy in diploid genome plus 5 μg rice genomic DNA from nontransformant was electrophoresed on lane C. The genomic DNAs from the six transformants are on lanes 1–6. All DNA was digested with *Bam*HI and hybridized with the maize genomic DNA probe (Fig. 1, probe A).

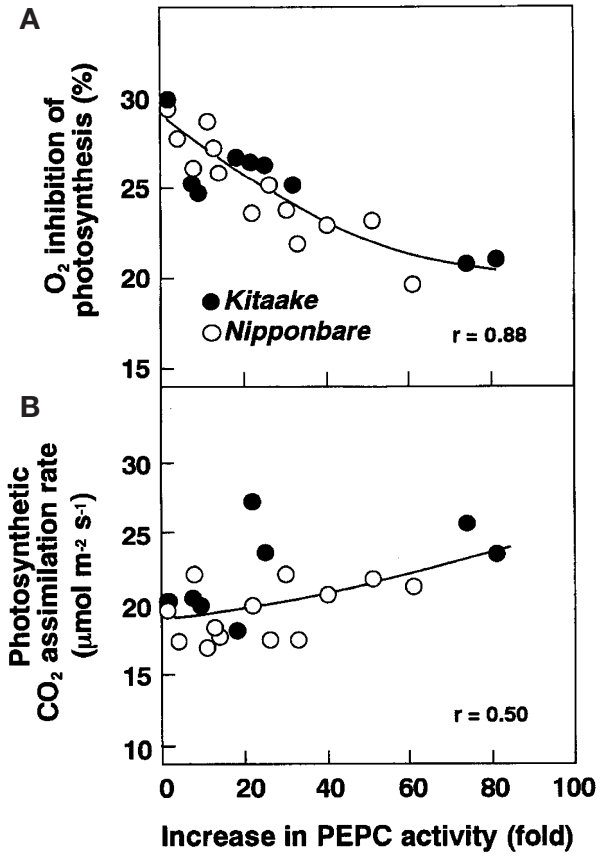

Figure 5. (A) Oxygen inhibition of photosynthesis for *Kitaake* (●), *Nipponbare* (○), and transgenic rice plants as a function of PEPC activity. Under similar conditions, maize leaves exhibit little or no apparent O_2 inhibition of photosynthesis[8,23]. (B) Photosynthetic rates of *Kitaake* and various transgenic rice plants, measured under ambient conditions.

The level of expression of maize PEPC in transgenic rice was correlated with the amount of the *pepc* mRNA and the copy number of the transgene. We investigated whether the high level expression of PEPC protein in transgenic rice plants was associated with the transcriptional activity of the introduced maize *pepc* gene. The level of the maize *pepc* transcript was tested in six primary transgenic rice plants with varying PEPC-specific activities. Among these plants, the amounts of PEPC polypeptide, as revealed by SDS-PAGE, correlated well with the enzyme activities (Fig. 4A). RNA gel blot analysis showed that the amount of steady-state *pepc* transcript also increased with increasing PEPC polypeptide in these plants (Fig. 4B). These results indicate that the high levels of expression were due to increased transcriptional activity.

We also carried out Southern blot analysis to estimate the copy number of the transgene in the transgenic rice plants. When the genomic DNAs of the transgenic rice plants digested with *Bam*HI were probed with a 1.0 kb maize genomic *Eco*RI fragment (Fig. 1, probe A), the probe hybridized with a 6.1 kb DNA fragment, and the intensity of hybridization increased with increasing amount of PEPC polypeptide among the six transgenic plants tested (Fig. 4C). The maize gene probe also hybridized with a 2.7 kb fragment of the rice genomic DNA, which probably encodes a rice *pepc* gene with a homologous sequence to the maize gene (Fig. 4C, lane R). To estimate the copy number of the transgene in the transgenic plants, a small amount of the plasmid DNA digested with *Bam*HI, which corresponded to the equivalent amount of one copy of diploid rice genomic DNA, was mixed with the *Bam*HI digested rice genomic DNA from untransformed plant, electrophoresed and hybridized with the maize gene probe (Fig. 4C, lane C). Based on the intensities of the 6.1 kb band in the six transgenic rice plants relative to that in lane C, we estimated the gene copy number in the transformants as one copy per diploid genome in the lane 1 plant, two in the lane 2 and lane 3 plants, three in the lane 4 plant, and more than four in the lane 5 and lane 6 plants. These results demonstrate that the level of expression of the maize PEPC in the transgenic rice plants depends on the gene copy number introduced. The results

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also demonstrate that the maize gene is actually transcribed in rice and synthesizes *pepc* mRNA comparable to that produced by the *pepc* gene in maize plant. In transgenic lane 2 and 3 rice plants, which contain one copy of the maize *pepc* gene in haploid genome, indeed have similar amounts of *pepc* mRNAs to that observed in maize (compare lane 2 or 3 with lane M, Fig. 4B).

Transgenic rice plants exhibited reduced O_2 inhibition of photosynthesis. High-level expression of the maize PEPC in transgenic rice plants resulted in considerable changes in the photosynthetic characteristic of these plants. O_2 inhibition of photosynthesis (as measured by CO_2 uptake) decreased progressively with increasing PEPC activity in the transgenic plants derived from both cultivars (Fig. 5A). As in other C_3 plants[22], atmospheric O_2 inhibits photosynthesis of untransformed *Kitaake* and *Nipponbare* by 30%, but this inhibition was reduced to about 20% in transgenic plants expressing high levels of maize PEPC. Under similar conditions, C_4 plants show little or no apparent O_2 inhibition of photosynthesis[7,23]. Under atmospheric conditions, the photosynthetic rates of transgenic plants were comparable to those of the two untransformed rice cultivars (Fig. 5B).

Discussion

In the present study, we introduced the intact gene encoding the maize C_4-specific PEPC into rice. The use of an intact gene was based on the findings of our previous studies that the promoters of maize genes encoding C_4 photosynthetic enzymes, such as PEPC and pyruvate, orthophosphate dikinase, can drive high levels of transcriptional activity of a reporter gene in transgenic rice plants[24,25]. The results of this study demonstrate that a gene involved in C_4 photosynthesis can be expressed in a C_3 plant (rice) at levels comparable to or even higher than those in C_4 plants, and the expressed C_4 enzyme remains functional (Fig. 2). In fact, the amount of the *pepc* mRNA transcribed from one copy of the transgene in haploid genome of transgenic rice plants is comparable to that produced from one *pepc* gene in maize plant (Fig. 4B and C). This is consistent with the notion that C_3 plants possess the necessary genetic machinery to express C_4-specific genes at high levels[7,24,25]. The maize gene was stably inherited in a Mendelian fashion and remained active in expressing the maize PEPC at high levels in the progenies (data not shown). The high activity of the maize enzyme is accompanied by a concomitant increase in protein, which accounts for up to 12% of the total leaf soluble protein in the transgenic plants (Fig. 3). To the best of our knowledge, such a high level of expression of a transgene in plants has not been achieved previously. So far, the highest level of expression of a transgene in plants was reported by Eckes et al.[26], who found that the alfalfa glutamine synthetase accounted for 5% of the total soluble protein in the leaves of transgenic tobacco plants. Furthermore, the high level of expression of the maize *pepc* gene in transgenic rice plants did not interfere with the growth and fertility of most of these plants.

Many factors can contribute to the overall expression of a transgene[27–31]. These include construct (transcriptional promoter and terminator, intron, and polyadenylation signal), codon usage, vector, method of gene transfer, tissue culture (selection and regeneration), site and copy number of gene integration, and degree of silencing at the transcriptional and post-transcriptional levels. Our results clearly show that one of the critical factors for the high-level expression of maize PEPC in rice is the copy number of the inserted gene. A higher copy number leads to a higher transcription and a higher amount of the enzyme. The use of an intact gene could be another reason for the high level expression of the maize PEPC in transgenic rice in the present study. The strength of the maize *pepc* promoter could also, in part, be responsible for the high level expression, as demonstrated in earlier studies in which it had been shown to drive high levels of expression of a reporter gene in stable transgenic rice plants[24,25]. Although the 35S promoter of cauliflower mosaic virus is a strong promoter, many transgenes under its transcriptional control exhibited silencing phenomenon[31] due to its viral nature, which may be considered invasive by the plants, and consequently triggers the plant's protective mechanisms for genome integrity. The presence of introns in the maize *pepc* gene used in this study could have contributed to the high-level expression, too. It has been reported that the presence of introns in transcription units, especially for transformation of monocots, can drastically enhance gene expression via an increase in the steady-state level of mRNA (e.g., the expression of maize sucrose synthase gene in maize[32]). In addition, the use of an intact monocot (e.g., maize) gene for transformation of a monocot plant (e.g., rice) may also have avoided the problem associated with pre-mRNA splicing. Several introns of monocot origin are known to be inefficiently processed in dicot cells[33,34]. In contrast to the high level of expression of maize PEPC obtained in this study, only low levels of expression were reported in transgenic tobacco plants transformed with an intact maize *pepc* gene[14] and in transgenic potato plants harboring a bacterial *pepc* gene[16]. These results, taken together, suggest that higher plants may be capable of expressing transgenes from closely related plants at high levels.

The high-level expression of a transgene in transgenic plants we have obtained in this study has important implications for molecular farming regarding production of useful proteins. Transgenic plants offer many production advantages as "protein factories." Plants should offer a high level of purity to avoid the contamination problems associated with the use of bacteria and animals as the source of protein production. It has been pointed out that the major technical challenge facing plant transformation biology today is the development of methods and constructs to produce a high proportion of plants showing predictable transgene expression without collateral genetic damage[27]. The methodology we employed in this study allowed us to produce transgenic plants with high levels of expression of a transgene without collateral damage.

The large variation in expression level of the maize PEPC in transgenic rice plants provides a good opportunity for examining the potential impact of maize PEPC on the carbon metabolism, physiology, and growth of rice plants in the future. This would also allow the screening of useful transformants with a desirable phenotype. The transgenic rice plants expressing high levels of the maize PEPC showed reduced sensitivity of photosynthesis to O_2 inhibition in a PEPC activity–dependent manner (Fig. 5). This could be due to a partial direct fixation of atmospheric CO_2 via enhanced maize PEPC, which is insensitive to O_2 inhibition. In addition, overexpression of the maize PEPC in rice leaves could change the carbon metabolism and partitioning of photoassimilates and thus sensitivity to O_2 (refs. 35 and 36). Further study is needed to address the biochemical basis for the reduced sensitivity of photosynthesis to O_2 and the effects of overexpression of the maize PEPC on the growth of transgenic rice plants.

Experimental protocol

Maize *pepc* gene. The intact maize gene encoding C_4-specific PEPC used for transformation is an 8.8 kb fragment containing all exons, introns, and the promoter (from –1212) and terminator (2.5 kb) sequences[37].

Tissue culture and rice transformation. Procedures for rice tissue culture and transformation with *Agrobacterium tumefaciens* were described previously[18]. Two Japonica rice cultivars (*Kitaake* and *Nipponbare*) were used for transformation.

Plant growth conditions. Both untransformed and transgenic plants were grown in a controlled environment room under a 14 h light (26°C) and 10 h dark (20°C) cycle. The photosynthetic photon flux density, provided by a combination of mercury and high pressure sodium lamps, was 500–600 μmol photon/m^2/s.

Extraction of leaf protein, assay of PEPC activity, gel electrophoresis, and

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western blot analysis. About 0.1 g leaf tissue was harvested from the mid-portion of four to six newly mature leaves from each plant in the light and quickly ground in 1.5 ml extraction buffer using cold mortar and pestle. The extraction buffer contained 50 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 5% insoluble polyvinylpolypyrrolidone and 10% glycerol. After total maceration, the crude extract was centrifuged at 15,000 G for 10 min, and the supernatant was used immediately for assay of PEPC. PEPC activity was assayed spectrophotometrically at room temperature in a mixture containing 50 mM tricine-KOH, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 3 U NAD-malate dehydrogenase, 0.2 mM NADH, and 0.01 ml protein extract (0.03 mg protein)[38]. The reaction was initiated by adding phospho*enol*pyruvate to a final concentration of 2 mM. For electrophoretic analysis, leaf soluble protein was mixed with a sodium dodecyl sulfate buffer and separated in a 7.5–15% linear gradient gel, as described previously[39]. For western blot analysis, protein in the gel was electrophoretically transferred onto a nitrocellulose membrane and probed with a rabbit antimaize PEPC serum (kindly provided by R. Chollet, University of Nebraska, Lincoln, NE). A goat antirabbit IgG conjugated to alkaline phosphatase was used to detect the maize PEPC. Protein concentration was determined using Bio-Rad (Hercules, CA) protein assay reagent.

Southern blot and RNA gel blot analyses. Genomic DNA was prepared from newly mature leaves by the method with cetyltrimethylammonium bromide[40]. For Southern blot analysis, genomic DNA (5 µg) was digested with *Bam*HI, and the resultant fragments were separated by electrophoresis on a 0.7% agarose gel and transferred onto a nylon membrane by an alkaline transfer method according to the manufacturer's instructions (Pall BioSupport, Glen Cover, NY). The membrane was hybridized with ^{32}P-labeled maize genomic DNA probe (a 1.0 kb *Eco*RI fragment, see probe A of Fig. 1), washed with 0.2×SSC and 0.1% SDS at 50°C for 1 h, and exposed to an image plate and analyzed using Fuji Film BAS2000.

Total RNA was isolated from newly mature leaves by the standard method with guanidine thiocyanate[41]. Total RNA (5 µg) was separated by electrophoresis and transferred onto a nylon membrane. The membrane was hybridized with ^{32}P-labeled maize PEPC cDNA (2.6 kb *Bam*HI fragment), washed with 0.2 ×SSC and 0.1% SDS at 50°C for 1 h, and exposed to an image plate.

Photosynthesis measurement. Photosynthetic CO_2 assimilation was measured at 30°C, 1200 µmol photon/m^2/s, 360 µl/L CO_2 and 2% or 21% O_2. The middle sections (3 cm) of four or five newly expanded leaves from each plant were sealed in the leaf cuvette, which was connected to an Analytical Development (Hoddesdon, UK) infrared gas analyzer and a Bingham Interspace (Hyde Park, NY) model BI-6-dp computer controller system[8]. The leaf cuvette contained a dew point sensor for measuring humidity and a copper-constantan thermocouple for monitoring leaf temperature. The gas exchange system was operated in an open mode and measured steady-state rates of CO_2 uptake. Percent O_2 inhibition of photosynthesis was calculated as (rate at 2% O_2 – rate 21% O_2) × 100/rate at 2% O_2.

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