Changes in Photosynthetic Carbon Flow in Transgenic Rice Plants That Express C4-Type Phospho*enol*pyruvate Carboxykinase from *Urochloa panicoides*

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A cDNA encoding phospho*enol*pyruvate carboxykinase (PCK) of *Urochloa panicoides* (a PCK-type C4 plant) was expressed in rice (*Oryza sativa* cv Tsukinohikari) plants under the control of the promoter of a maize (*Zea mays*) gene for phospho*enol*-pyruvate carboxylase or pyruvate, orthophosphate dikinase with the transit peptide of the small subunit of Rubisco. Crude extracts prepared from the green leaves of transgenic plants had high PCK activity and the newly expressed PCK was localized in chloroplasts. In labeling experiments with $^{14}CO_2$ up to 20% of the radioactivity was incorporated into 4C compounds (malate, oxaloacetate, and aspartate) in excised leaves of transgenic plants, as compared with about 1% in excised leaves of control plants. There was a positive correlation between PCK activity and the extent of labeling of 4C compounds. When L-[4-^{14}C]malate was fed to excised leaves the extent of incorporation of radioactivity into sucrose was 3-fold greater in transgenic plants than in control plants and the level of radiolabeled aspartate was significantly lower in transgenic plants. These results indicate that the ectopic expression of PCK in rice chloroplasts was able partially to change the carbon flow in mesophyll cells into a C4-like photosynthetic pathway. Such a strategy appears to provide a possible method for enhancing the photosynthetic capacity of C3 plants.

To generate varieties of rice (*Oryza sativa*) with enhanced ability to fix CO_2 we attempted to increase the concentration of CO_2 at the site of Rubisco by introducing a C4 photosynthetic decarboxylating enzyme, phospho*enol*pyruvate carboxykinase (PCK), into the chloroplasts of rice mesophyll cells. In PCK-type C4 plants, PCK [ATP: oxaloacetate carboxylase (transphosphorylase); EC 4.1.1.49 (ATP-dependent)] is a cytosolic enzyme in bundle sheath cells; it catalyzes the reversible decarboxylation of oxaloacetate (OAA) to phospho*enol*pyruvate (PEP). We postulated that if PCK expressed in chloroplasts decarboxylated OAA a concentration of CO_2 inside chloroplasts would be increased and the increase would cause more efficient fixation of CO_2 by Rubisco. Furthermore, concomitantly increased PEP would serve as a substrate for non-C4-type PEP carboxylase (PEPC) that is found in the cytosol of rice mesophyll cells. Thus, a CO_2-fixing cycle would be created between the cytosol and chloroplasts without a requirement for pyruvate, orthophosphate dikinase (PPDK). We attempted to determine whether CO_2 produced as a result of the activity of PCK might be routed to the photosynthetic fixation of carbon and, if such was the case, to determine whether transgenic rice with increased photosynthetic efficiency could be obtained by the additional expression of PEPC in the cytosol.

Rice is a C3 photosynthetic plant and the C4 photosynthetic pathway does not operate in any member of the genus *Oryza* (Yeo et al., 1994). It is difficult to introduce features of the C4 photosynthetic pathway into rice by conventional breeding. However, the availability of an efficient system for transformation of rice (Shimamoto et al., 1989; Hiei et al., 1994) suggests that genetic engineering should allow us to introduce the capacity for C4-type photosynthesis into rice plants. Several attempts to express C4 photosynthetic enzymes in C3 plants have been reported. cDNA or the gene for PEPC from various sources has been introduced into tobacco (Hudspeth et al., 1992; Kogami et al., 1994), potato (Gehlen et al., 1996), and rice (Ku et al., 1999), and the gene for PPDK has been introduced into Arabidopsis (Ishimaru et al., 1997). Although the photosynthetic characteristics of the transgenic plants differed from those of the parental plants, no enhancement of photosynthetic ability was observed in most cases. Transgenic rice plants transformed with an intact gene for C4-specific PEPC from maize (*Zea mays*) expressed PEPC at a remarkably high level in their leaves and their sensitivity to inhibition of photosynthesis by oxygen was reduced (Ku et al., 1999). 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 the light (Häusler et al., 1999). Lipka et al. (1999) reported

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recently that transgenic potato that expressed both the NADP-malic enzyme of *Flaveria pringlei*, a C3 plant, in chloroplasts and the PEPC of *C. glutamicum* in the cytosol had a reduced requirement for electrons for the assimilation of CO_2 in strong light and at high temperature.

Various cDNAs for PCK have been isolated from cucumber (Kim and Smith, 1994), oilseed rape (Saez-Vasquez et al., 1995), *Urochloa panicoides* (Finnegan and Burnell, 1995), and maize (Furumoto et al., 1999). However, only a cDNA for PCK of *U. panicoides* was available to us when we started the present work so we used this cDNA. Our analysis of transgenic rice plants revealed that CO_2 released in chloroplasts in the reaction catalyzed by PCK was rapidly incorporated into photosynthetic metabolites.

RESULTS

Transformation of Rice and Expression of PCK

Two types of PCK construct, pPKS and pDKS, each with a different promoter, were used for transformation. The presence of the transgenes and expression of PCK in green leaves was confirmed by PCR and western-blot analysis, respectively. Among 100 independent regenerated PKS lines, 47 contained the transgene and 24 expressed detectable PCK protein. In transformation experiments with pDKS we obtained 54 regenerated plants; 31 lines contained the transgene and 20 lines expressed detectable PCK protein. The level of PCK varied significantly among the transgenic lines, but there were no differences in levels of PCK between PKS and DKS lines. No immunostained protein and no PCK activity were detected in the leaves of non-transgenic lines, indicating that the green leaves of parental rice plants did not contain PCK at detectable levels.

The molecular mass of immunochemically detected PCK from green leaves of the transgenic lines was 62 kD and corresponded to that of the purified enzyme from *U. panicoides* (Finnegan and Burnell, 1995). It was smaller than the molecular mass of PCK with the transit peptide of Rubisco produced in transformed *Escherichia coli* cells. We calculated that the M_r of the chimeric PCK polypeptide, without the transit peptide, should be 62,730.

Some transgenic lines (R_0) were satisfactorily fertile and produced seeds (R_1). The PCK and PEPC activities in leaves of these R_1 plants are shown in Figure 1. The non-transgenic parent and segregated lines without a transgene (null transformants) had no detectable PCK activity. By contrast, the transgenic lines had significant, but varied levels of PCK activity, indicating that the PCK transgene had been expressed and that active PCK had been produced in the transgenic lines. All lines of non-transgenic and transgenic rice plants had low, but varied levels of PEPC activity, but there was no significant difference between controls and transgenic lines. When we compared PCK and PEPC activities among individual transgenic lines, we found no correlation between the activities of PCK and PEPC. When leaf extracts with identical PCK activity were fractionated by SDS-PAGE and stained immunochemically, the intensities of the bands of PCK from the transgenic lines and *U. panicoides* were similar, indicating that the specific activity of PCK that was expressed in the transgenic lines was almost the same as that in *U. panicoides* (data not shown). The photosynthetic characteristics such as net photosynthetic rate, CO_2 compensation points, the chlorophyll contents, and length of culms and panicles were not different between controls and transgenic lines (Table I).

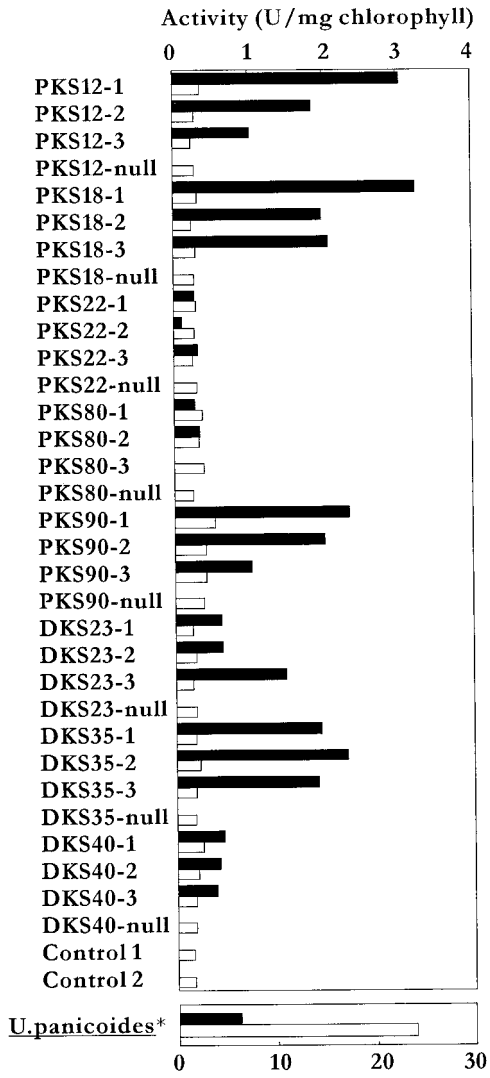

Figure 1. The activities of PCK and PEPC in various transgenic rice plants. The activities of PCK (black column) and PEPC (white column) in crude extract prepared from leaf-tip sections of transgenic rice plants (R_1 generation of PKS and DKS lines) were measured as described in the text. Transformants are numbered according to the code number for individual R_0 plants (first no.), which is followed by a second number that indicates the individual plant of R_1 generation derived from self-pollinated of R_0 plants. Asterisk, Burnell and Hatch (1988).

Intracellular Localization of PCK

Protoplasts isolated from leaf mesophyll cells of the transgenic line DKS35 contained a single immunoreactive polypeptide of 62 kD. A polypeptide of the same size was also found in intact chloroplasts regardless of whether or not they had been treated with trypsin (Fig. 2). By contrast, broken chloroplasts that had been treated with trypsin contained smaller polypeptides. Since C4-type PCK exists as a hexamer (Burnell, 1986), digestion by trypsin of the native enzyme was limited and incomplete under our conditions. Furthermore, since all of the 62-kD polypeptide was converted to smaller polypeptides upon treatment of broken chloroplasts, but not of intact chloroplasts with trypsin, it appeared that a significant amount of PCK derived from the transgene had been transported into chloroplasts as expected from the presence of the transit peptide.

Tracer Experiments with $^{14}CO_2$

To identify the metabolites generated immediately after fixation of CO_2, we applied a short pulse (5 s) of $^{14}CO_2$ to leaf-tip sections and analyzed soluble labeled metabolites by two-dimensional thin-layer chromatography (TLC). The incorporation of radioactivity into each identified component was expressed as a percentage of the total radioactivity in the soluble fraction (Table II). The incorporation of $^{14}CO_2$ into 4C compounds (malate, OAA, and Asp) in transgenic lines was 10-fold higher than that in control and null plants, whereas the incorporation of radioactivity into other labeled compounds did not differ significantly between transgenic and the non-transgenic plants with the exception that the rate for photosynthetic assimilates (3-phosphogycerate [3-PGA] and sugar phosphates) was slightly lower in the transgenic lines than in the control and null plants. The incorporation of radioactivity into 4C compounds was positively correlated with the PCK activity in the transgenic lines (Fig. 3).

Table I. *Photosynthetic and agronomic traits of transgenic rice plants*

Net photosynthetic rate (A) and CO_2 compensation point (Γ) were measured using R_1 and R_2 generations of 8- to 10-week-old PKS lines, respectively. Chlorophyll content, culm length, and panicle length were taken from R_1 generation of PKS lines. Data show the means ± SD of results from four or five separate experiments. All data were not significantly different between transformants and control plants by Student's *t* test.

Trait	Transformant	Control
A (μmol CO_2 m^{-2} s^{-1})	29.5 ± 2.4	28.2 ± 2.2
Γ (μl L^{-1})	53.9 ± 3.0	52.3 ± 2.8
Chlorophyll content (mg/g fresh wt)	3.92 ± 0.82	4.12 ± 0.93
Culm length (cm)	60.0 ± 8.2	64.5 ± 9.7
Panicle length (cm)	14.9 ± 2.5	16.5 ± 2.2

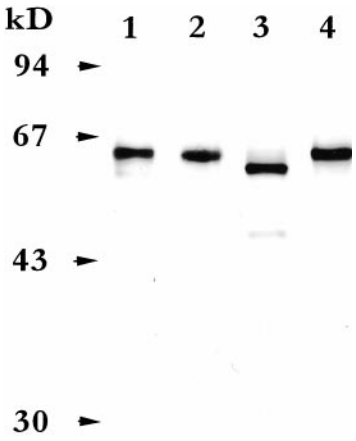

Figure 2. Treatment with trypsin of chloroplasts isolated from green leaves of transgenic rice plants (R_3 generation of DKS35). Isolated intact chloroplasts were broken by freezing and thawing and intact and broken chloroplasts were incubated with 50 μg mL^{-1} trypsin. Proteins were then separated by SDS-PAGE and PCK was detected by western blotting. Lane 1, Protoplasts; lane 2, intact chloroplasts after treatment with trypsin; lane 3, broken chloroplasts after treatment with trypsin; lane 4, intact chloroplasts without exposure to trypsin. Arrowheads indicate mobilities of marker proteins.

The increase in incorporation of CO_2 into 4C compounds in the transgenic lines might have resulted from the reaction catalyzed by PEPC and/or from the reversible reaction catalyzed by PCK. To determine whether the increase was due to endogenous PEPC activity we examined the effects of a specific inhibitor of PEPC, namely, 3,3-dichloro-2-(dihydroxyphosphinyl-methyl)-propenoate (DCDP). When leaf-tip sections were treated with 5 mM DCDP before the 5-s pulse of $^{14}CO_2$, the rate of incorporation of $^{14}CO_2$ into 4C compounds was significantly reduced in the transgenic line, whereas the incorporation of radioactivity into other metabolites was the same in transgenic and control plants (Fig. 4). The incorporation of radioactivity into intermediates of photorespiration (Ser, Gly, glycerate, and glycolate) was increased by the treatment with DCDP prior to the pulse of $^{14}CO_2$ in both transgenic and control plants.

The above results suggested that the increased incorporation of CO_2 into 4C compounds might have been caused by endogenous PEPC activity. However, the activity of PEPC measured in leaf extracts did not differ between the transgenic and control plants. Apparently, elevated PCK activity in the transgenic plants led to increased incorporation of CO_2 into 4C compounds and, thus, it seemed likely that the elevated PCK was responsible for the increased supply of PEP as a substrate for PEPC. To examine whether an elevated cellular level of PEP could affect the rate of incorporation of CO_2 into 4C compounds, we fed PEP to leaf-tip sections before pulse feeding with $^{14}CO_2$. Prior incubation of leaf-tip sections with PEP more than doubled the incorporation of $^{14}CO_2$ in control plants, but not in transgenic plants (Fig. 5). Theses results indicated that the activity of PEPC was

Table II. *Relative levels of labeled metabolites after a 5-s pulse of $^{14}CO_2$*

Leaf-tip sections of transgenic rice plants of the R_2 generation and control rice plants were exposed to a 5-s pulse of $^{14}CO_2$ as described in the text. Radiolabeled metabolites were separated by two-dimensional TLC and radioactivity of identified spots was measured with a Bioimage Analyzer (BAS 1000, Fuju Film, Tokyo). The relative levels of each metabolite are expressed as percentages of total radioactivity. DKS35-1 and DKS35-2 were individual plants derived from the same R_0 plant. PKS12 and PKS12-null (null-transformant) were derived from same R_0 plant and segregated in the R_1 generation. The sum of each column is less than 100% because the compounds in a small number of spots were unidentified.

Metabolite	Plant Material				Control
	DKS35-1	DKS35-2	PKS12	PKS12-null	
Malate + OAA[a]	7.8	5.9	8.8	0.8	0.8
3-PGA + Sugar-P[a]	65.2	69.2	53.5	75.9	77.8
Suc	8.5	9.0	9.7	9.9	9.4
PEP	0.8	1.2	0.5	1.1	1.1
Asp	6.1	4.8	6.9	0.7	0.7
Ser	1.3	0.4	2.3	1.3	0.9
Gly	3.1	1.4	4.5	3.4	3.1
Ala	1.2	3.0	1.8	0.7	0.7
Glycerate	2.5	0.6	5.0	2.1	1.4
Glycolate	2.0	4.0	4.0	3.0	3.0

[a] Not separatable by two-dimensional TLC.

limited by the low level of its substrate and that, in the PCK transgenic rice, PCK supplied increased amounts of PEP to PEPC, thereby increasing the rate of conversion of PEP to OAA and the incorporation of $^{14}CO_2$.

If 4C compounds were decarboxylated by PCK in transgenic rice, we would expect the radioactivity in 4C compounds to decrease during the chase period in transgenic rice, but not in control plants. Nearly one-half of the radioactivity that had initially been fixed in 4C compounds disappeared during the chase with $^{12}CO_2$ and radioactivity was apparently transferred to other metabolites in the transgenic rice. By contrast, most of the radioactivity in 4C compounds remained in these compounds in the control plants (Fig. 6). Since

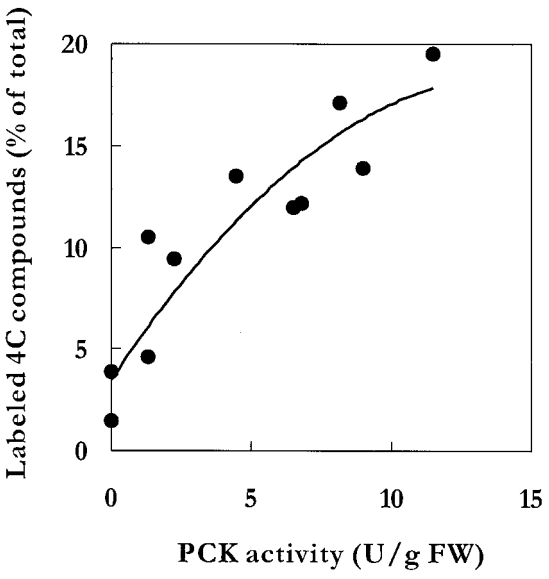

Figure 3. Relationship between PCK activity and the accumulation of ^{14}C-labeled 4C compounds in leaves of transgenic rice plants. PCK activities and relative levels of ^{14}C-labeled 4C compounds (malate, OAA, and Asp) that accumulated after a 5-s pulse of $^{14}CO_2$ were determined for the same transgenic rice plants (R_1 generations of DKS or PKS lines). The data obtained from several transformants with different PCK activities are plotted on the graph.

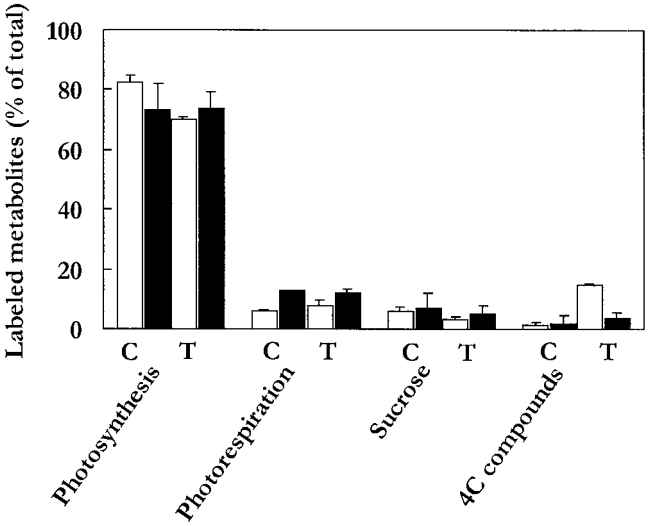

Figure 4. The effects of treatment with DCDP on the subsequent incorporation of $^{14}CO_2$. Detached leaf-tip sections of control (C; non-transformants) and transformants (T; R_1 generation of DKS35) were placed in 10 mM sodium phosphate buffer (pH 6.4) with (solid bars) or without (open bars) 5 mM DCDP. After illumination for 30 min, a 5-s pulse of $^{14}CO_2$ was applied. ^{14}C-labeled compounds were identified and categorized as follows: photosynthesis (3-PGA and sugar phosphates); photorespiration (Ser, Gly, glycerate, and glycorate); Suc; and 4C compounds (malate, OAA, and Asp). Each value indicates the relative level (percentage of total radioactivity) of labeled metabolites. Each column and bar show the mean + SE of results from four individual experiments.

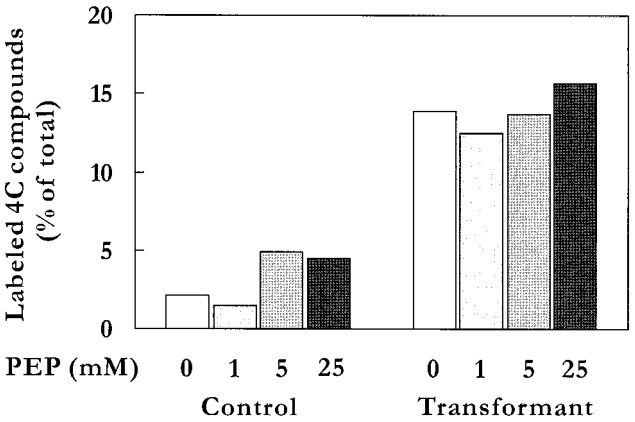

Figure 5. Effects of loading with PEP on the relative levels of radiolabeled 4C compounds. Detached leaf-tip sections of control (non-transformant) and transformant (R_1 generation of DKS35) plants were placed in a solution that contained 0, 1, 5, or 25 mM PEP (as indicated by shading). After illumination for 30 min, a 5-s pulse of $^{14}CO_2$ was applied. The relative levels of ^{14}C-labeled 4C compounds (malate, OAA, and Asp) were compared among sections. Each column shows the mean of results from two individual experiments.

the extent of labeling of 4C compounds was low and the C3 photosynthetic fixation of CO_2 contributed to much stronger radiolabeling of other metabolites, no increase in the radioactivity of photosynthetic assimilates that corresponded to the decrease in radioactivity of 4C compounds was detected. When we repeated these experiments with other transgenic lines, we found similar changes in the radioactivity of 4C compounds in every case.

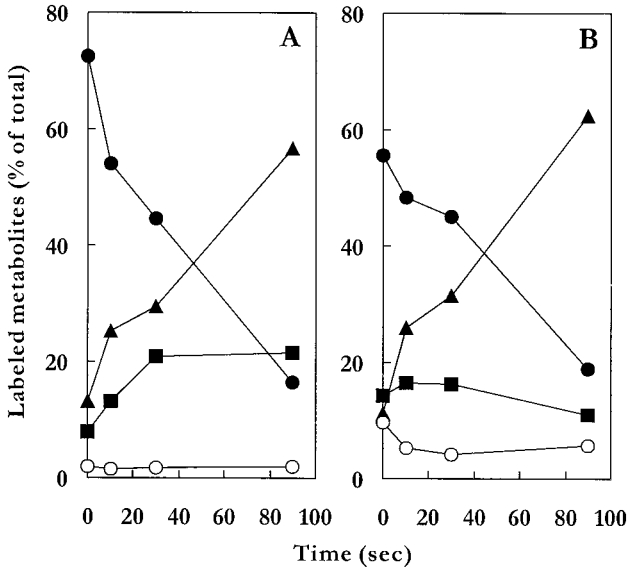

Figure 6. Changes in rates of labeling of photosynthetic metabolites after a 5-s pulse of $^{14}CO_2$ and a chase with $^{12}CO_2$. Detached leaf-tip sections of control (A; non-transformants) or transgenic (B; R_1 generation of DKS35) rice were exposed to a 5-s pulse of $^{14}CO_2$ that was followed by a $^{12}CO_2$ chase for 10, 30, and 90 s. ^{14}C-labeled metabolites were identified and categorized as follows: products of photoassimilation (●), 3-PGA and sugar phosphates; photorespiratory intermediates (■), Ser, Gly, glycerate and glycolate; Suc (▲); and 4C compounds (○), malate, OAA, and Asp.

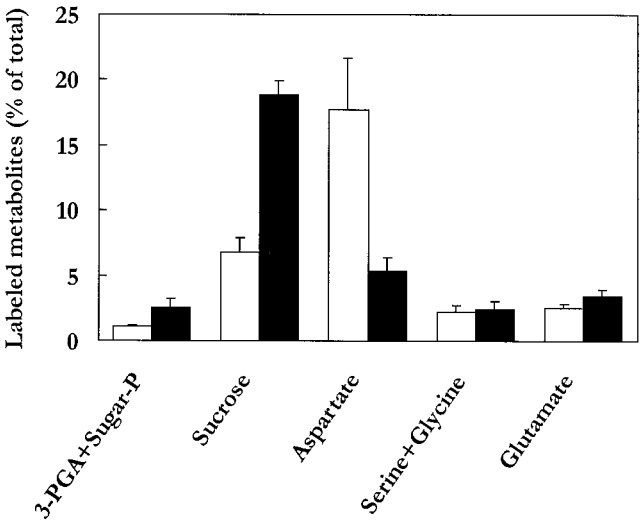

Figure 7. Comparison of the relative levels of labeled metabolites after feeding of leaf-tip sections with L-[4-^{14}C]malate. After illumination for 60 min, leaf-tip sections of control (white bars) and transgenic (black bars; R_1 generation of DKS35) rice were placed in a solution that contained 2.2×10^4 Bq of L-[4-^{14}C]malate. After 15 min, ^{14}C-labeled compounds were isolated and identified. Each column and bar shows the means + SE of results from four individual experiments.

Tracer Experiments with L-[4-^{14}C]Malate

To confirm that CO_2 released by PCK was fixed in photosynthetic assimilates, we fed L-[4-^{14}C]malate to leaf-tip sections for 15 min and then examined the radiolabeled metabolites. PCK decarboxylates the 4-C carboxyl group of OAA that is synthesized from malate in vivo without changing the position of the other carboxyl group. Thus if PCK of transgenic rice were to function as expected, the incorporation of ^{14}C during a relatively short period of time into photosynthetic assimilates should be much greater in transgenic rice than in the control plants. The incorporation of ^{14}C from L-[4-^{14}C]malate into 3-PGA plus sugar phosphates and Suc was 3-fold higher in transgenic leaves than in the controls (Fig. 7). By contrast, the incorporation of ^{14}C into Asp was greatly reduced, whereas that into photorespiratory intermediates (Ser and Gly) and Glu remained unchanged. The incorporation of radioactivity into photoassimilates was light-dependent; the rate of incorporation of ^{14}C in darkness was very low, with little or no difference between the rates of incorporation between transgenic and control plants (data not shown).

DISCUSSION

In this study we introduced cDNA for a C4-type PCK into the chloroplasts of rice plants under the control of the promoter of the maize gene for PEPC or

PPDK and then we examined the effects of the expressed PCK on the rate of reassimilation of fixed CO_2. When we generated the PCK cDNA constructs used in this work, only two clones (λPCK1100101 and λPCK170204) of PCK cDNA from *U. panicoides* were available, and we fused these cDNAs to obtain a cDNA that encoded an N-terminally truncated PCK. The nucleotide sequences of the two cDNA fragments revealed that although λPCK1100101 encoded the C-terminal region that included the codon for termination of translation, λPCK170204 encoded an incomplete N-terminal region that lacked the initiation codon ATG (Finnegan et al., 1999). However, the latter sequence did include a coding region that corresponded to the N-terminal amino acid sequence of the purified active enzyme, even though it was still truncated (Finnegan and Burnell, 1995). Our fused cDNA construct corresponded to the coding sequence of purified truncated PCK, starting with Ser-57, and the product had the appropriate enzymatic activity. It was reported similarly that partial proteolysis of PCK at the N-terminal end did not affect the V_{max} of the reaction catalyzed by purified PCK from cucumber (Walker and Leegood, 1995). Finnegan et al. (1999) showed recently that *U. panicoides* has four genes for PCK (*PCK 1–PCK 4*), with λPCK1100101 corresponding to *PCK 1* and λPCK170204 to *PCK 2*. The amino acid sequences encoded by the coding regions of *PCK 1* and *PCK 2* were 96% identical.

The transgenic lines of rice that harbored the fused PCK cDNA with the transit peptide of rbcS gave similar results in terms of levels of expression of PCK and the metabolism of 4C compounds, regardless of the source of the promoter. Although the fused cDNA turned out to be a hybrid of cDNAs for *PCK 1* and *PCK 2*, the product of cDNA in transgenic rice had PCK activity and the specific activity of the PCK was similar to that of PCK from *U. panicoides*. Kinetic analysis of the partially purified enzyme from one line of transgenic rice indicated that the reaction catalyzed by PCK proceeded toward the decarboxylation of OAA, and rates of CO_2 exchange with OAA and carboxylation of PEP were negligible (data not shown). The transgenes and their ability to express PCK were stably inherited to the R_3 generation. Furthermore, the agronomic traits of the transgenic lines were similar to the control plants (Table I).

Labeling experiments with $^{14}CO_2$ revealed that the rate of fixation of CO_2 into 4C compounds via the reaction catalyzed by PEPC was significantly increased in the transgenic rice plants. Moreover, such increases were correlated with the activity of PCK, whereas the activity in vitro of PEPC in extracts of the transgenic plants remained low and was similar to or only slightly higher to that in control plants. DCDP, a selective inhibitor of PEPC, inhibited the incorporation of radioactivity into 4C compounds. Thus, the increased fixation of CO_2 must have been due to the action of endogenous PEPC. These results suggest that PCK expressed in rice chloroplasts might have generated a supply of PEP from chloroplasts to the cytosol where endogenous PEPC, activated by this increased supply of its substrate, enhanced the incorporation of CO_2 into 4C compounds. Indeed, treatment of leaf-tip sections with exogenous PEP to elevate cytosolic levels of PEP subsequently increased the relative level of radioactive 4C compounds in control plants, but not in transgenic plants (Fig. 5). However, even through incorporation was enhanced, the incorporation of $^{14}CO_2$ by PEP-treated control plants did not reach the levels observed in transgenic plants.

The exogenous PEP was supplied through the cut ends of leaves. Thus, transport to and uptake of PEP by cells might have been very limited. Therefore, a small but significant increase in the cytosolic level of PEP would be expected to increase the incorporation of $^{14}CO_2$. By contrast, cytosolic PEP in transgenic rice must have been supplied efficiently to PEPC as a consequence of the elevated activity of PCK. Thus, a small increase in the level of PEP due to the exogenous supply of PEP should not affect the rate of incorporation of $^{14}CO_2$. A novel plastidic phosphate transporter that exchanges PEP for inorganic phosphate has been identified in C3 and C4 plants (Fischer et al., 1997). Therefore, the presence of PCK in chloroplasts appears to have generated a PEP/OAA cycle between the cytosol and chloroplasts in the transgenic rice. The reason for the small but significant increase in the amount of ^{14}C in photorespiratory intermediates in DCDP-treated rice plants is not clear.

We performed two types of experiments to demonstrate that CO_2 released from OAA by PCK is fixed into photosynthetic assimilates. In the pulse-chase experiment we found that the radioactivity in 4C compounds after a pulse of $^{14}CO_2$ decreased rapidly during the chase period in the transgenic lines, but not in the control plants. The ^{14}C-labeled OAA that is first generated by PEPC contains a radiolabeled carbon atom at the C-4 position and is converted to malate and Asp. Malate is also decarboxylated directly by malic enzyme. However, if the release of CO_2 upon the direct decarboxylation of malate had occurred to any significant extent, a rapid decrease in radioactivity of 4C compounds should have occurred in the control plants, but such a decrease did not in fact occur. In the transgenic rice by contrast, PCK in chloroplasts directly decarboxylated OAA with a resultant rapid decline in radioactivity. In the second type of experiment when L-[4-^{14}C]malate was fed to leaf sections, the relative rates of incorporation into Suc and into 3-PGA plus sugar phosphates in the transgenic lines were 3- and 2-fold higher, respectively, than in the control plants, whereas the rate of incorporation into Asp was greatly reduced in the transgenic plants. These results indicate that, since L-[4-^{14}C]malate is easily converted to [4-^{14}C]OAA in

vivo, CO_2 generated from the C-4 carbon atom of OAA by introduced PCK was efficiently fixed into photosynthetic assimilates. Incorporation of ^{14}C of L-[4-^{14}C]malate into the photosynthetic assimilates in the control plants should have occurred via decarboxylation of intermediates formed from L-[4-^{14}C]malate in the tricarboxylic acid cycle. The decreased rate of incorporation of radioactivity into Asp in the transgenic rice suggests that PCK competed effectively with Asp aminotransferase for available OAA.

The evidence obtained in this work suggests that, in our lines of PCK transgenic rice, some fixation of CO_2 was catalyzed by endogenous cytosolic PEPC. The resultant OAA was decarboxylated by the product of the transgene, PCK, in chloroplasts and the CO_2 that was released was refixed via the photosynthetic carbon-reduction cycle. Finally, the PEP generated was recruited by cytosolic PEPC. However, the contribution of this C4-like carbon cycle to the photosynthetic fixation of carbon was very limited, probably because of the low level of PCK expressed in chloroplasts and the low level of endogenous PEPC. No significant differences in the net photosynthetic rate and the CO_2 compensation point were observed between the transgenic lines and the control plants (Table I). The C4-like fixation of CO_2, operating between the cytosol and chloroplasts in a manner analogous to the compartmentalization of C4 mesophyll and bundle sheath cells, respectively, might endow C3 plants with unique photosynthetic characteristics if C4-type PEPC and PCK could be expressed at high levels similar to those in C4 plants and located in the cytosol and chloroplasts, respectively. A remarkably high level of expression of maize PEPC in rice has been achieved by introducing an intact gene for maize PEPC (Ku et al., 1999), and we are currently exploring ways to increase the level of expression of PEPC in rice. It seems that the coordinated and high-level expression of carboxylating and decarboxylating enzymes is critical for an efficient C4-like carbon cycle.

Another point that requires attention is the transport of 4C compounds and PEP across the envelope membrane of the chloroplast. Efficient transport of PEP might be achieved by the plastidic phosphate transporter that is present in both C3 and C4 plants (Fischer et al., 1997). Although dicarboxylate translocators import C4 acids into chloroplasts, they are antiporters that require another C4 acid (Flügge, 1998) and are not suitable for the continuous operation of a C4-like carbon cycle. Engineering of the translocators might allow us to overcome this problem. Our observations that rice plants were able to decarboxylate 4C compounds and that the rate of carboxylation of PEP was not affected by preloading with PEP indicate that an efficient mechanism exists for the transport of 4C acids into rice chloroplasts and that an efficient mechanism also exists for the transport of PEP out of the chloroplasts.

MATERIALS AND METHODS

Chemicals

DCDP was custom-synthesized, as described in a previous report (Jenkins et al., 1987), by Wako Pure Chemical Industries (Osaka). L-[4-^{14}C]Malate was prepared with maize (*Zea mays*) PEPC (Wako Pure Chemical) as described previously (Hatch, 1972). $NaH^{14}CO_3$ was obtained from Amersham (Buckinghamshire, UK).

Construction of Plasmid Vectors

The promoter regions of the maize genes for PEPC and PPDK and the sequence encoding the transit peptide region of the small subunit of rice (*Oryza sativa*) Rubisco (rbcS-TP) were isolated by PCR. Primers for amplification of each respective DNA fragment were synthesized as described in previous reports (and rbcS-TP, Matsuoka et al., 1988; PEPC, Hudspeth and Grula, 1989; PPDK, Glackin and Grula, 1990). Amplified DNA fragments corresponding to 702 bp of the promoter region of the gene for PEPC (−571 to 131 relative to the site of initiation of transcription) and 954 bp of the promoter region of the gene for PPDK (−789 to 165) were cloned into pCR1000 (Invitrogen, Carlsbad, CA). Plasmids harboring individual amplified fragments in the correct orientation were digested with *Kpn*I and *Eco*RI (PEPC promoter) or *Hin*dIII and *Eco*RI (PPDK promoter), and the resultant fragments were inserted into the multicloning site of plasmid pBIISK$^-$ (Stratagene, La Jolla, CA). The cloned promoter for maize PPDK was digested with *Sac*I (at 67 relative to the site of initiation of transcription), blunt-ended with T4 DNA polymerase, and modified by addition of an *Nco*I linker at the blunt end. The 150-bp rbcS-TP fragment was modified by addition of *Eco*RI-*Nco*I and *Xba*I sites at the 5′ and 3′ terminus, respectively, and then it was inserted into the *Eco*RI/*Xba*I sites of pUC18 to yield pTP1. The sequence between the processing site and the *Xba*I site had been modified such that the fusion with PCK cDNA was in-frame.

The sequence encoding PCK was constructed from two cDNA fragments that encoded parts of the PCK of *Urochloa panicoides*, namely, λPCK1100101 (Finnegan and Burnell, 1995) and λPCK170204 (Finnegan et al., 1999). λPCK170204 and λPCK1100101 encoded the N-terminal region and the C-terminal region, respectively, and the encoded region overlapped in the middle region of PCK. Although λPCK170204 encoded the N-terminal amino acid sequence of the purified enzyme, which exhibited enzymatic activity but lacked the first 56 amino acids of the intact PCK polypeptide (Finnegan and Burnell, 1995), it lacked part of the 5′-coding region (Finnegan et al., 1999). λPCK170204 was digested with *Kpn*I and the resultant 900-bp fragment was ligated to the *Kpn*I site of λPCK100101, which encoded the C-terminal one-half of the PCK polypeptide. The fused cDNA included the entire coding region, starting with the N-terminal Ser-57 residue of the purified active enzyme, and the translated product of the fused cDNA had PCK activity in transgenic rice plants (see below). *Xba*I and *Sph*I sites were added to the 5′ and 3′ terminus of the fused cDNA, respec-

tively, by PCR and after digestion with the corresponding restriction enzymes, the fused cDNA was inserted to the *Xba*I/*Sph*I sites of pUC18. Then the 150-bp *Eco*RI/*Xba*I fragment encoding rbcS-TP, excised from pTP1, was inserted at the *Eco*RI/*Xba*I sites of the resultant plasmid to yield pTP-PCK.

An *Nco*I/*Eco*RI fragment of pGL2 (Biland et al., 1991) containing the cauliflower mosaic virus 35S terminator region was inserted at the *Nco*I/*Eco*RI site of the plasmids that contained the promoter region of the maize PEPC gene (700 bp) or the maize PPDK gene (860 bp). The resultant respective plasmids were digested with *Nco*I and *Sph*I and the 2.2-kbp *Nco*I/SphI fragment excised from pTP-PCK was inserted into the plasmids. The plasmids harboring the PEPC promoter::PCKcDNA::35S terminator construct and the PPDK promoter::PCKcDNA::35S terminator construct were designated pPKS and pDKS, respectively.

Transformation

Transgenic rice plants were generated as reported previously (Shimamoto et al., 1989). Protoplasts were prepared from suspension-cultured cells derived from scutella of immature seeds of japonica rice (cv Tsukinohikari). pDKS or pPKS and pGL2, which included a gene for hygromycin phosphotransferase, were introduced together into protoplasts by electroporation. Regenerated plantlets (R_0 plants) were transferred to paddy soil in pots once they had reached 10 cm in height and were grown in a greenhouse (28°C day/23°C night). Transgenic rice plants harboring pDKS and pPKS, respectively, were designated DKS and PKS lines.

Assay of Enzymatic Activity

For measurements of enzymatic activities, 7-cm-long leaf-tip sections were excised from fully expanded and non-senescent leaves of R_1 plants that had been grown for 1 month. Leaf-tip sections (approximately 0.1 g) were homogenized with a mortar and pestle in 1 mL of extraction buffer (2 mM $MnCl_2$, 2 mM $MgCl_2$, 1 mM EDTA, 0.1% [v/v] 2-mercaptoethanol, and 10% [v/v] glycerol in 50 mM HEPES (2-[4-(hydroxyethyl)-1-piperazinyl] ethanesulfonic acid)-KOH, pH 7.0) that contained 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM 6-amino-*n*-caproic acid, 0.2% (w/v) sodium isoascorbate, and 2% (w/v) Polyclar AT (Gokyo Sangyou, Osaka). The homogenate was centrifuged at 17,000*g* for 15 min and the supernatant was desalted on a NAP5 column (Pharmacia, Uppsala) that had been previously equilibrated with the extraction buffer. The desalted crude extract was used as the solution of enzymes for measurements of PCK and PEPC activities. The amount of chlorophyll in the homogenate was measured basically as described by Wintermans and De Mots (1965).

The PCK activity in extracts was determined as described by Burnell (1986). Each reaction mixture contained 25 mM HEPES-KOH buffer (pH 8.0), 4 mM dithiothreitol, 0.5 mM OAA, 2 mM $MnCl_2$, 1 unit of pyruvate kinase (Boehringer Mannheim, Mannheim, Germany), and 25 μL of the enzyme solution in a total volume of 1 mL. PCK activity was measured by following the ATP-dependent (0.2 mM ATP) change in absorbance of OAA at 280 nm. The PEPC activity was measured in 1 mL of a mixture of 4 mM dithiothreitol, 5 mM $KHCO_3$, 5 mM $MgSO_4$, 1 mM Glc-6-P, 0.25 mM NADH, 5 mM PEP and 4 units of malate dehydrogenase (Boehringer Mannheim) in 25 mM HEPES-KOH buffer, pH 8.0. The reaction was started by addition of 25 μL of the enzyme solution to the mixture and the reaction was monitored spectrophotometrically at 340 nm. One unit of enzymatic activity was defined the amount of enzyme that generated 1 μmol of product per minute at 25°C.

Intracellular Localization of PCK

Mesophyll protoplasts were isolated from the leaves of 10-d-old seedlings of transgenic rice (R_3 generation of DKS transformants) as described by Toyama et al. (1989). Intact chloroplasts were prepared from protoplasts as described by Edwards et al. (1978) and suspended in chloroplast buffer (50 mM HEPES-KOH, pH 8.0, 1 mM $MgCl_2$, 5 mM EDTA, and 0.33 M sorbitol). Broken chloroplasts were prepared by freezing and thawing intact chloroplasts in the same buffer without sorbitol. The preparations of intact and broken chloroplasts were treated with trypsin (Wako Pure Chemical) at 50 μg mL^{-1} for 20 min on ice and then trypsin inhibitor (type IV-0; Sigma, St. Louis) was added at 0.5 mg mL^{-1}. Trypsin-treated intact chloroplasts were pelleted by centrifugation at 1,800*g* for 30 s, resuspended in 50 mM HEPES-KOH buffer (pH 8.0), frozen, and thawed. Soluble fractions of chloroplast samples were prepared by centrifugation and subjected to western-blot analysis, as described below.

Detection of PCK by Western-Blot Analysis

Proteins were extracted from leaves by homogenization in loading buffer for SDS-PAGE (Laemmli, 1970). Samples were fractionated by SDS-PAGE (10% [w/v] polyacrylamide) and electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated with rabbit antiserum against PCK from *U. panicoides* (Finnegan and Burnell, 1995). Goat antibodies against rabbit IgG, conjugated with horseradish peroxidase, were used as the second antibodies. Blots were developed by 4-chloro-1-naphthol and hydrogen peroxide.

Gas Exchange Measurement

Net photosynthetic rate (A) and CO_2 compensation point (Γ) of rice plants were measured with an infrared gas analyzer (model LI-6400, LI-COR, Lincoln, NE). The conditions in the assimilation chamber were kept as follows: air humidity, 70%; leaf temperature, 30°C; light intensity, 1,000 μmol m^{-2} s^{-1}. Measurement was carried out using middle portion (3 cm long) of the fully expanded and not senescent leaf blade. Net photosynthetic rate was taken at the CO_2 concentration of 350 μL L^{-1}. CO_2 compensation

point was estimated from the data of net photosynthetic rate at various CO_2 concentrations.

Tracer Experiments

Five-centimeter-long leaf-tip sections of fully expanded and non-senescent leaves were placed upright with cut ends immersed in distilled water in a glass chamber (air space of approximately 50 mL) and air was passed continuously through the chamber at a flow rate of 5 L min^{-1}. Illumination at 400 μmol m^{-2} s^{-1} was provided by a metal halide lamp [DR400/TL(L); Toshiba, Tokyo]. After illumination for 30 min, the air flow was changed to the circulation mode, and 9×10^5 Bq of $^{14}CO_2$ were injected into the chamber. After pulse feeding for 5 s, the leaf sections were quickly removed from the chamber and plunged into liquid nitrogen within 2 s. For the "chase" experiment, the air flow was changed back to the original pass-through mode and incubation was continued. At various times after the 5-sec pulse of $^{14}CO_2$, individual leaf sections were removed and plunged into liquid nitrogen as described above. In some experiments DCDP or PEP was fed to leaf-tip sections via the cut-ends during the initial 30-min illumination. $^{14}CO_2$ was generated by mixing 100 μL of 60% (w/w) perchloric acid and 1.9×10^6 Bq $NaH^{14}CO_3$ (2×10^9 Bq/mmol) in a gas-tight syringe and an aliquot of $^{14}CO_2$ corresponding to 9×10^5 Bq was withdrawn using a syringe.

For feeding experiments with L-[4-^{14}C]malate, the cut ends of leaf-tip sections that had been illuminated as described above for 1 h were immersed in 2.2×10^4 Bq of L-[4-^{14}C]malate (2×10^9 Bq/mmol) in 100 μL of 10 mM phosphate buffer (pH 6.4) and incubated for a further 15 min under illumination.

Soluble metabolites in leaf-tip sections were extracted with 80% (v/v) hot ethanol. Each extract was concentrated in vacuo and subjected to two-dimensional TLC on a cellulose thin-layer plate (Funacel SF, Funakoshi Company, Tokyo). A mixture of phenol:water:acetic acid:0.5 M EDTA (47:84:5.5:1.14, v/v) was used as the mobile phase in the first dimension and a mixture (1:1, v/v) of 1-butanol:water (74:5, v/v) and propionic acid:water (9:11, v/v) was used as the mobile phase in the second dimension. The TLC plate was exposed to an imaging plate and the radioactivity in each spot was quantified with a Bioimage Analyzer. Radioactive metabolites were identified by reference to the mobilities of standard compounds.

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