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CO_2 DONATION BY MALATE AND ASPARTATE REDUCES PHOTORESPIRATION IN *Panicum milioides*, A C_3-C_4 INTERMEDIATE SPECIES*

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SUMMARY: Oxygen inhibition of leaf slice photosynthesis in *Panicum milioides* increased from 20% to 30% at 21% O_2 in the presence of maleate, a phosphoenolpyruvate carboxylase inhibitor. The increased O_2 sensitivity was completely reversed by the addition of malate and aspartate, the stable products of the phosphoenolpyruvate carboxylase reaction. The C_4 acids, malate and aspartate, also reduced O_2 inhibition of photosynthesis by isolated bundle sheath strands, but not mesophyll protoplasts. Similarly, only bundle sheath strands exhibited an active C_4 acid-dependent O_2 evolution. Compartmentation of C_4 cycle enzymes, with pyruvate, Pi dikinase in the mesophyll and NAD-malic enzyme in the bundle sheath, was demonstrated. It is concluded that reduced photorespiration in *P. milioides* is due to a limited potential for C_4 photosynthesis permitting an increase in pCO_2 at the site of bundle sheath ribulosebisphosphate carboxylase.

Panicum milioides, a naturally occurring species with Kranz-like leaf anatomy, is intermediate between C_3 and C_4 plants with respect to photorespiration (1-6). Two major hypotheses have been proposed for this intermediacy: a significant refixation of photorespired CO_2 by PEP† carboxylase before it exits the leaf (2,3); and the presence of an altered Rbu-P_2 carboxylase-oxygenase with an increased affinity for CO_2 relative to O_2 (4).

Since *P. milioides* represents the first well documented higher plant species with reduced photorespiration, it is of considerable importance to determine the biochemical mechanism(s) involved. Recently we provided evidence for the participation of PEP carboxylase, rather than an altered

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†Abbreviations: PEP, phosphoenolpyruvate; Rbu-P_2, ribulose 1,5-bisphosphate; Rib-5-P, ribose 5-phosphate; Rbu-5-P, ribulose 5-phosphate; α-Kg, α-ketoglutarate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; C_4 acids, malate and aspartate; Chl, chlorophyll.

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Rbu-P_2 carboxylase-oxygenase, in reducing O_2 inhibition of net photosynthesis in *P. milioides* (6). In this paper we report that a limited C_4-like CO_2 pump, mediated by a PEP carboxylation-C_4 acid decarboxylation reaction sequence similar to that in NAD-malic enzyme-type C_4 plants (7-9), is responsible for the reduced photorespiration and O_2 sensitivity of net photosynthesis in *P. milioides*.

MATERIALS AND METHODS

Growth conditions for *P. milioides* and the methods for preparing leaf slices were as previously described (6,10). Leaf mesophyll protoplasts and bundle sheath strands were isolated by digesting leaf slices at 37°C with an enzyme medium similar to that of Kanai and Edwards (11) except that 4% (w/v) Meicelase P cellulase (Meiji Seika Kaisha Ltd., Tokyo) replaced Onozuka cellulase and pectinase was omitted. Leaf and cell extracts were prepared at 4°C as described previously (6), and enzyme activities were determined at 30°C according to established procedures (6,12).

Light-dependent $^{14}CO_2$ fixation experiments were run at 30°C in serum-stoppered vials containing a standard reaction mixture of 0.3 M sorbitol, 50 mM Tricine, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM KH_2PO_4 and 3 mM *iso*ascorbate adjusted to pH 8.0 (6). Assay conditions were similar to (6). Light-dependent O_2 evolution was measured polarographically at 30°C under CO_2-free conditions (8). Other details are described in the text.

RESULTS AND DISCUSSION

We have recently shown that in addition to PEP carboxylase, other diagnostic enzymes of C_4 photosynthesis such as pyruvate, Pi dikinase and NAD-malic enzyme are also present at a reduced level of activity in leaves of *P. milioides* (6). We also provided evidence for an NAD-malic enzyme-catalyzed decarboxylation of malate and aspartate at the C-4 carboxyl position by *P. milioides* leaf slices (6). These observations suggested that perhaps photorespiration and the associated O_2 inhibition of photosynthesis is reduced in *P. milioides* by a limited C_4-like PEP carboxylation-C_4 acid decarboxylation system permitting an increase in pCO_2 at the site of Rbu-P_2 carboxylase-oxygenase (6).

An important feature of C_4 photosynthesis is the compartmentation of carbon metabolism between mesophyll and bundle sheath cells (7). The conventional Onozuka cellulase-pectinase enzyme digestion system that has been developed for isolating pure cell types from leaves of C_4 plants (8, 10,11,

13) yielded cross-contaminated mesophyll and bundle sheath preparations from *P. milioides*. Therefore, we developed an enzyme digestion procedure using Meicelase for isolating pure mesophyll protoplasts and bundle sheath strands from leaves of *P. milioides*, as judged by light microscopy. Complete details of the isolation and purification procedures will be described elsewhere.

Table I shows the activity and intercellular distribution of key photosynthetic and photorespiratory enzymes between mesophyll and bundle sheath cells of *P. milioides*. The specific activity of PEP carboxylase, the first enzyme of the carboxylative phase of the C_4 pathway (7), is 4-times greater in the mesophyll than in the bundle sheath. Pyruvate, Pi dikinase, catalyzing PEP regeneration from pyruvate, is exclusively localized in mesophyll cells. Another biochemical feature associated with C_4 photosynthesis is the decarboxylation of C_4 acids in the bundle sheath (7-9). Of the three known C_4 acid decarboxylating enzymes (NADP- and NAD-malic enzymes and PEP carboxykinase) present in leaves of C_4 plants (7-9), only NAD-malic enzyme activity was detected in leaf extracts of *P. milioides* (6) and this activity was exclusively localized in the bundle sheath cells (Table I). An approximately equal distribution of aminotransferases and NAD-malate dehydrogenase between the two cell types of *P. milioides* (on a specific activity basis) further suggests a compartmentation of C_4 pathway enzymes similar to that in NAD-malic enzyme-type C_4 plants (7,9). Consistent with recent *in situ* immunofluorescent localization studies of Rbu-P_2 carboxylase in *P. milioides* leaf sections (14), the *in vitro* enzyme data in Table I indicate that several marker enzymes of the Calvin cycle and photorespiration are present in both cell types, with the bundle sheath having about 3-times the specific activity of the mesophyll. Independent experiments supporting this intercellular distribution of C_4- and C_3-photosynthetic enzymes based on exogenous substrate effects on light-dependent $^{14}CO_2$ fixation by the isolated cell types and the exclusive localization of $/\overline{4}$-$^{14}\underline{C}/C_4$ acid decarboxylation in intact bundle sheath strands will be reported elsewhere.

TABLE I. Activity and Distribution of Enzymes Associated with C_4- and C_3-Photosynthesis and Photorespiration between Mesophyll Protoplasts and Bundle Sheath Strands of *Panicum milioides*

Enzyme	Specific Activity (μmol/mg Chl·hr^{-1})		
	Whole Leaves	Mesophyll Protoplasts	Bundle Sheath Strands
C_4-Photosynthesis			
PEP carboxylase	131	180	45
Pyruvate, Pi dikinase	109	180	0
NAD-malic enzyme	83	0	153
Aspartate aminotransferase	756	529	965
Alanine aminotransferase	276	271	271
NAD-malate dehydrogenase	1436	1305	1871
C_3-Photosynthesis			
Rbu-P_2 carboxylase	506	174	596
NADP-triose-P dehydrogenase	624	292	748
Rib-5-P isomerase/Rbu-5-P kinase	704	365	1219
Photorespiration			
P-Glycolate phosphatase	498	222	818
Glycolate oxidase	67	29	81
Glycine decarboxylase	46	21	65
Hydroxypyruvate reductase	477	154	825
Chl *a:b* ratio	2.74	2.89	2.37

The importance of thin leaf slices in studying regulatory aspects of photorespiratory and photosynthetic carbon metabolism during the coordinated functioning of mesophyll and bundle sheath cells and the specific inhibition of PEP carboxylase by maleate under the conditions employed have recently been documented (6,10). Figure 1A shows the effect of 2% and 21% O_2 on photosynthetic $^{14}CO_2$ fixation by leaf slices of *P. milioides* in the absence and presence of maleate at low HCO_3^- (0.48 mM, 10.5 μM CO_2 at pH 8.0, 30°C) as a function of exogenous malate plus aspartate concentration. Photosynthesis by control leaf slices of *P. milioides* (without maleate) was inhibited 20% at 21% O_2 and exogenous C_4 acids had no effect on either the rate of $^{14}CO_2$ fixation or the percent inhibition by 21% O_2. In the presence of saturating levels of maleate (5 mM, Refs. 6,10) the rate of $^{14}CO_2$ fixation was decreased 20% at 2% O_2 and 30% at 21% O_2. This reduction in photosynthesis was associated with a concomitant increase in the O_2 inhibition of net photosynthesis from 20% in control leaf slices to 30% by specific PEP carboxylase inhibition, a value identical to that observed with *P. scribnerianum*, a

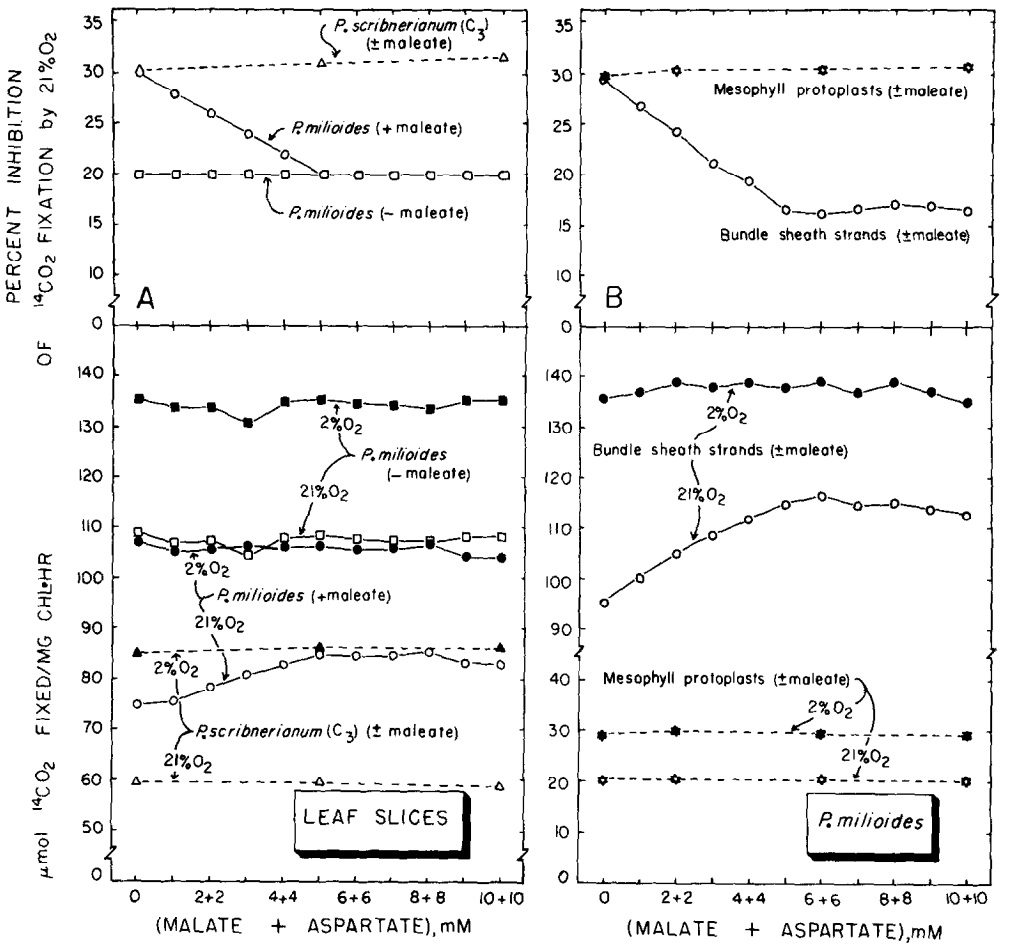

FIG. 1. Effect of exogenous malate plus aspartate concentration on the rate of light-dependent $^{14}CO_2$ fixation by leaf slices (A) and isolated mesophyll protoplasts and bundle sheath strands (B) of *P. milioides* at 30°C, 0.48 mM $NaH^{14}CO_3$ (10.5 µM CO_2 at pH 8.0) and 2% or 21% O_2 in the absence and presence of 5 mM maleate. Fig. 1A also includes, for comparison, studies with leaf slices of field-grown *P. scribnerianum*, a C_3 species (15). Percent inhibition by 21% O_2 (upper panels) was calculated from the observed rates of $^{14}CO_2$ fixation at 2% O_2 versus 21% O_2. The leaf slices were preincubated in the standard assay medium (± maleate and/or C_4 acids) for 30-40 min, and flushed with the appropriate O_2/N_2 mixture for 10-15 min before the reaction was initiated with $NaH^{14}CO_3$ (6). α-Kg (10 mM) was always included with aspartate. Light intensity, 5 to 10.5 x 10^3 µE $m^{-2}.s^{-1}$ (400-700 nm).

representative C_3 species (Fig. 1A). While exogenous C_4 acids had no effect on $^{14}CO_2$ fixation by maleate-treated leaf slices at 2% O_2, the rate at 21% O_2 gradually increased with increasing levels of C_4 acids. The observed increase at saturating C_4 acid concentration was about 14%. Figure 1A further docu-

ments the complete reversal of the increased O_2 sensitivity of photosynthesis in maleate-treated leaf slices to a value identical to that observed in control leaf slices (without maleate) by saturating levels of C_4 acids. Under identical conditions, neither maleate nor exogenous C_4 acids had any effect on the rate of $^{14}CO_2$ fixation at 2% or 21% O_2 or on O_2 inhibition of photosynthesis in *P. scribnerianum* leaf slices (Fig. 1A).

Since *P. milioides* leaves have Kranz-like anatomy (1,16) with NAD-malic enzyme, the C_4 acid decarboxylase, exclusively localized in the bundle sheath (Table I), the effect of exogenous C_4 acids on isolated mesophyll protoplast and bundle sheath strand photosynthesis was examined (Fig. 1B). In bundle sheath strands the inhibition of $^{14}CO_2$ fixation by 21% O_2 was 30%. While exogenous C_4 acids had no effect on photosynthesis at 2% O_2, the rate at 21% O_2 gradually increased with increasing C_4 acid concentration. The observed increase at saturating levels of C_4 acids was about 20%. More noteworthy, the C_4 acids reduced the extent of O_2 inhibition of bundle sheath photosynthesis by about 45%. In the absence of exogenous C_4 cycle substrates mesophyll protoplasts fixed $^{14}CO_2$ at a relatively low rate, in agreement with the reduced activity of Calvin cycle enzymes in this cell type (Table I). The inhibition of mesophyll photosynthesis by 21% O_2 was 30%, and the O_2 sensitivity was unaffected by all C_4 acid concentrations examined (Fig. 1B), consistent with the absence of a C_4 acid decarboxylation system (Table I).

The observed O_2 sensitivity of leaf slice and isolated cell photosynthesis was a typical Warburg effect in that the O_2 inhibition at low HCO_3^- (0.48 mM) was completely overcome at high HCO_3^- levels (10 mM). Maximum C_4 acid reversal of O_2 inhibition at low HCO_3^- was observed when both malate and aspartate were added together rather than singly. No effect of exogenous C_4 acids on O_2 sensitivity of leaf slice (maleate-treated) or bundle sheath strand (± maleate) photosynthesis was observed at 2 mM HCO_3^- (cf., Fig. 1A,B), suggesting that CO_2 donation by the C_4 acids was responsible for the partial reversal of O_2 inhibition at 0.48 mM HCO_3^-.

TABLE II. Substrate Requirements for Oxygen Evolution by Illuminated Mesophyll Protoplasts and Bundle Sheath Strands of *P. milioides*

Substrate	μmol O_2 evolved/mg Chl·hr^{-1}	
	Mesophyll Protoplasts	Bundle Sheath Strands
HCO_3^- (10 mM)	52.6	134
Aspartate + α-Kg + malate*	0	45.6
+ NH_4Cl (20 mM)	-	0
+ DCMU (10 μM)	-	0
Darkness	-	0
Aspartate + α-Kg	0	37.1
Malate + α-Kg	0	14.3
Aspartate + malate	0	17.1
Aspartate	0	16.4
Malate	0	14.3
α-Kg	-	0

*Concentrations of added compounds were: aspartate, 10 mM; α-Kg, 10 mM; malate, 10 mM. Light intensity, 1.1 to 1.4 x 10^3 μE $m^{-2}·s^{-1}$ (400-700 nm).

When malate plus aspartate were added to the isolated cell types, only bundle sheath strands exhibited an active light-dependent O_2 evolution, the rate being one-third of that observed at saturating HCO_3^- (Table II). The suggestion that the C_4 acid-dependent O_2 evolution was due to CO_2 donation and fixation via the Calvin cycle rather than photoreduction of oxaloacetate, an intermediate formed during aspartate decarboxylation (7-9), was confirmed by the complete inhibition of O_2 evolution by the photophosphorylation uncoupler, NH_4Cl. Radiotracer kinetic studies designed to investigate the labeling of C_4 acids during short-term photosynthesis in $^{14}CO_2$ and the transfer of ^{14}C from the C-4 carboxyl of C_4 acids to Calvin cycle intermediates during $^{14}CO_2$ pulse-chase experiments with whole leaves, leaf slices and isolated cell types of *P. milioides* are currently in progress.

The observations described in this paper indicate that the reduced O_2 inhibition of net photosynthesis in *P. milioides* is due to PEP carboxylase participation since specific inhibition of this enzyme by maleate results in a typical C_3 O_2 sensitivity (Fig. 1A, Ref. 6). The function of PEP carboxylase is not simply to refix photorespired CO_2 as previously suggested (2,3),

since the addition of malate and aspartate, the stable products of PEP carboxylation, to maleate-treated (ie., PEP carboxylase-inhibited) leaf slices completely reverses the increased O_2 sensitivity back to the original reduced value (Fig. 1A). Experiments with the isolated cell types (Fig. 1B, Table II) indicate that the C_4 acids donate CO_2 only to the bundle sheath Calvin cycle, consistent with the exclusive localization of the C_4 acid decarboxylase in this cell type (Table I). The present study thus provides the first indication that the C_3-C_4 intermediate nature of *P. milioides* with respect to photorespiration is due to the potential for a limited C_4-like PEP carboxylation-C_4 acid decarboxylation system permitting an increase in pCO_2 at the site of bundle sheath Rbu-P_2 carboxylase-oxygenase.

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