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Photosynthetic Characteristics of *Portulaca grandiflora*, a Succulent C_4 Dicot[1]

CELLULAR COMPARTMENTATION OF ENZYMES AND ACID METABOLISM

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ABSTRACT

The succulent, cylindrical leaves of the C_4 dicot *Portulaca grandiflora* possess three distinct green cell types: bundle sheath cells (BSC) in radial arrangement around the vascular bundles; mesophyll cells (MC) in an outer layer adjacent to the BSC; and water storage cells (WSC) in the leaf center. Unlike typical Kranz leaf anatomy, the MC do not surround the bundle sheath tissue but occur only in the area between the bundle sheath and the epidermis. Intercellular localization of photosynthetic enzymes was characterized using protoplasts isolated enzymatically from all three green cell types.

Like other C_4 plants, *P. grandiflora* has ribulose 1,5-bisphosphate carboxylase and the decarboxylating enzyme, $NADP^+$-malic enzyme, in the BSC. Unlike other C_4 plants, however, phosphoenolpyruvate carboxylase, pyruvate, Pi dikinase, and $NADP^+$-malate dehydrogenase of the C_4 pathway were present in all three green cell types, indicating that all are capable of fixing CO_2 via phosphoenolpyruvate carboxylase and regenerating phosphoenolpyruvate. Other enzymes were about equally distributed between MC and BSC similar to other C_4 plants. The enzyme profile of the WSC was similar to that of the MC but with reduced activity in most enzymes, except mitochondrion-associated enzymes.

Intracellular localization of enzymes was studied in organelles partitioned by differential centrifugation using mechanically ruptured mesophyll and bundle sheath protoplasts. Phosphoenolpyruvate carboxylase was a cytosolic enzyme in both cells; whereas, ribulose 1,5-bisphosphate carboxylase and $NADP^+$-malic enzyme were exclusively compartmentalized in the bundle sheath chloroplasts. $NADP^+$-malate dehydrogenase, pyruvate, Pi dikinase, aspartate aminotransferase, 3-phosphoglycerate kinase, and $NADP^+$-triose-P dehydrogenase were predominantly localized in the chloroplasts while alanine aminotransferase and NAD^+-malate dehydrogenase were mainly present in the cytosol of both cell types. Based on enzyme localization, a scheme of C_4 photosynthesis in *P. grandiflora* is proposed.

Well-watered plants of *P. grandiflora* exhibit a diurnal fluctuation of total titratable acidity, with an amplitude of 61 and 54 microequivalent per gram fresh weight for the leaves and stems, respectively. These changes were in parallel with changes in malic acid concentration in these tissues. Under severe drought conditions, diurnal changes in both titratable acidity and malic acid concentration in both leaves and stems were much reduced. However, another C_4 dicot *Amaranthus graecizans* (nonsucculent) did not show any diurnal acid fluctuation under the same conditions. These results confirm the suggestion made by Koch and Kennedy (Plant Physiol. 65: 193–197, 1980) that succulent C_4 dicots can exhibit an acid metabolism similar to Crassulacean acid metabolism plants in certain environments.

Plants possessing the C_4-dicarboxylic acid metabolism of photosynthesis are characterized anatomically by having two photosynthetic cell types in the leaves, mesophyll cells and distinct bundle sheath cells (Kranz type leaf anatomy). The structure and biochemical function of these two types of leaf cells in C_4 plants have been of much scientific interest over the past decade (3, 17, 20). Variations in leaf anatomy have been observed among C_4 plants (17, 19, 21). The succulent dicot *Portulaca grandiflora* Hook has been reported as a C_4 plant based on a measurement of $\delta\ ^{13}C$ (24) and biochemical studies (7). Anatomically, the succulent cylindrical leaves of *P. grandiflora*, in addition to mesophyll and bundle sheath cells, have a third distinct type of green cell which is localized in the center of the leaves. These cells form the water tissue and contain scattered chloroplasts. There are many morphological and anatomical similarities between succulent C_4 dicots and CAM plants. Recently, it has been reported that under certain environmental conditions, *Portulaca oleracea*, a succulent C_4 dicot, is capable of developing an acid metabolism with many similarities to CAM (13, 14). In this report, leaf anatomy and acid metabolism were studied in *P. grandiflora*. Protoplasts from the three cell types were isolated and key photosynthetic enzymes were located to determine their potential function in C_4 photosynthesis. The results indicate that the succulent C_4 dicot *P. grandiflora* has a slightly different Kranz leaf anatomy and enzyme compartmentation compared to other C_4 plants and that it is also capable of an acid metabolism similar to that of CAM plants.

MATERIALS AND METHODS

Plant Materials. Plants of *P. grandiflora* Hook were grown from seeds in a greenhouse under a day/night temperature regime of 25 to 30/20 to 25 C and a light period of 15 h. Light was provided with Lucolux lamps (General Electric, Cleveland, OH) giving a range of quantum flux density between 100 and 120 $nE/cm^2 \cdot s$ on the canopy. The plants were watered with diluted Hoagland's nutrient solution twice a week. Young and newly expanded leaves from 6- to 8-week-old plants were used for protoplast isolation. In the study of titratable acidity and malate concentration in leaf and stem tissues some of the *P. grandiflora* plants were subjected to

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drought conditions by withholding water. Two other species, *Amaranthus graecizans* (C_4) and *Portulacaria afra* (CAM) were included for comparisons. Plants of these two species were propagated from cuttings and were cultivated under the same conditions as *P. grandiflora*.

Leaf Cross Section. Leaf material for transverse cross section was fixed with fluoroacetic acid and embedded in JB4 plastic. Sections of 4 μm were cut with a microtome and stained with 1% aniline B.

Protoplast Isolation. Leaves were cut perpendicular to the long axis into 1-mm slices with a razor blade. For the isolation of protoplasts from WSP[3], leaf slices were incubated in an enzyme medium containing 0.6 M sorbitol, 2% (w/v) cellulase (Onozuka R-10), 0.5% (w/v) macerozyme (Onozuka R-10), 0.1% (w/v) BSA, 5 mM Mes-KOH (pH 5.1) and 1 mM $CaCl_2$. After 1–2 h digestion at 30 C and under low light (20 nE/cm^2·s), the mixture was washed with a buffer solution containing 0.6 M sorbitol, 5 mM Hepes-KOH (pH 7.0), and 1 mM $CaCl_2$ and passed through a 150 μm nylon net. The filtrate was stored on ice, allowed to settle, and the pellet formed was used for protoplast purification. Longer digestion periods were avoided to eliminate contamination of WSP preparations by MP which usually released with further digestion time.

For the isolation of MP and BSP, leaf slices were incubated in a similar enzyme medium except that the osmoticum was reduced to 0.3 M sorbitol. The osmoticum of the buffer solution for washing also was reduced. After 3 to 6 h digestion, the mixture was washed and passed through a 105 μm nylon net. The filtrate was stored on ice, and the pellet formed was used for protoplast purification and separation. Under these conditions, WSP burst during incubation and isolation. Increasing the osmoticum of the enzyme medium resulted in a lower yield of BSP.

Purification and separation of protoplasts from different types of leaf cells were achieved according to Edwards *et al* (6), except that the osmoticum of the purification and separation system was 0.6 M sucrose for the WSP and 0.5 M sucrose for the MP and BSP. After purification with the sucrose-dextran system, the mixture of MP and BSP was resuspended in a sorbitol medium containing 0.5 M sorbitol, 5 mM Hepes-KOH (pH 7.0), and 1 mM $CaCl_2$. About 2 ml of this protoplast suspension was overlayered onto 10 ml separation medium consisting of 0.5 M sucrose, 5 mM Hepes-KOH (pH 7.0), and 1 mM $CaCl_2$. After centrifugation at 300*g* for 2 min, the MP partitioned at the interface between the sorbitol and sucrose layer while the BSP sedimented to the bottom of the test tube. The MP was collected with a Pasteur pipette and resuspended in the sorbitol medium. The BSP pellet was resuspended in the sorbitol medium and the separation was repeated.

Preparation of Protoplast and Whole Leaf Extracts. After purification and separation, the protoplasts were resuspended in a breaking medium containing 0.3 M sorbitol, 50 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM cysteine, and 1% (w/v) PVP-40 and ruptured by gentle dispersion several times through a 50-μl microsyringe. The protoplast extracts were then subjected to differential centrifugation for organelle separation. The whole leaf extract was made by grinding 2 g of leaf segments in a Ten-Broeck homogenizer (Corning, Houghton Park, NY) containing 8 ml of 50 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM cysteine, and 2.5% (w/v) PVP-40. The homogenate was transferred to a test tube and centrifuged at 10,000*g* for 10 min. The supernatant was used for enzyme assays. To prepare the extracts to assay pyruvate, Pi dikinase, the breaking medium for protoplasts and the extraction medium for whole leaf tissues included 1.5 mM sodium pyruvate in addition to the components mentioned above.

Enzyme Assay and Chl Determination. The following enzymes were assayed as previously described: RuBP carboxylase (18), PEP carboxylase, $NADP^+$-malic enzyme, NAD^+-malate dehydrogenase, and $NADP^+$-malate dehydrogenase (12), PEP carboxykinase (5), NAD^+-malic enzyme (10), PGA kinase, and $NADP^+$-triose-P dehydrogenase, Cyt *c* oxidase, and catalase (16), alanine aminotransferase, and aspartate aminotransferase (1). The spectrophotometric assay of 3-PGA kinase was coupled to triose-P dehydrogenase using either NADH or NADPH as a cofactor. Four-fold higher activities were obtained with NADH as the cofactor. Pyruvate, Pi dikinase was assayed spectrophotometrically by measuring the decrease in A_{340} in a reaction medium containing 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 1.5 mM sodium pyruvate, 2.5 mM K_2HPO_4, 10 mM NH_4Cl, 25 mM $NaHCO_3$, 1.25 mM ATP, 0.2 mM NADH, 1.5 units of malate dehydrogenase (Sigma), and 1.5 units of purified crabgrass PEP carboxylase in a total volume of 1.0 ml. The reaction was initiated by adding ATP. All extracts, except for the assay of Cyt *c* oxidase, were supplemented with Triton X-100 to a final concentration of 0.05% prior to assay. Chl was determined according to Wintermans and De Mots after extraction in 96% (v/v) ethanol (26).

Measurements of Titratable Acidity and Malate Concentration. Titratable acidity and malate concentration of leaf and stem tissues were measured at the beginning and the end of the photoperiod. One-half gram sample was removed from the plants, sliced into small segments, and ground completely in a Ten-Broeck homogenizer containing 15 ml distilled H_2O. The crude homogenate was transferred to a test tube and boiled for 10 min. The extract was cooled to room temperature and centrifuged at 10,000*g* for 10 min. An aliquot of the supernatant was titrated with 4 mM KOH to pH 8.3. The data are expressed as μeq of acid/g fresh weight.

Malic acid was assayed spectrophotometrically by measuring the reduction of NADPH at 340 nm in a reaction coupled to NADP-malic enzyme (Sigma). The reaction mixture contained 50 mM Mops (pH 7.4), 5 mM $MgCl_2$, 1 mM $NADP^+$, 1 unit of malic enzyme, and 50–100 μl of the extract in a final volume of 1.0 ml.

RESULTS

Leaf Anatomy. The C_4 dicot *P. grandiflora* possesses cylindrical, succulent leaves. Three distinct types of chlorenchymatous cells can be recognized in a tranverse cross section of a leaf: (a) bundle sheath cells in radial arrangement around the vascular bundles; (b) mesophyll cells in the outer layer adjacent to the bundle sheath cells; and (c) water tissue in the center of the leaf (Fig. 1). The bundle sheath cells contain many large chloroplasts concentrated in a centripetal position. Unlike typical Kranz leaf anatomy, the mesophyll cells do not completely surround the bundle sheath tissue, but occur only in the area between the bundle sheath and the epidermis. The large cells of the water tissue contain large vacuoles and only a few chloroplasts.

Protoplast Isolation. For the isolation of MP and BSP, an osmoticum of 0.3 M sorbitol during digestion resulted in a higher yield of both protoplast types than with higher osmotica. However, 0.5 M sorbitol was required to stabilize the protoplasts during purification. Under these conditions, WSP burst. The WSP were more stable in 0.6 M sorbitol so it was used throughout the course of isolation.

Figure 2 shows preparations of isolated MP, BSP, and WSP of *P. grandiflora*. These three protoplast types are readily distinguished under the light microscope based on their size and chloroplast density and orientation. The BSP are generally larger and contain more chloroplasts than those of the MP. The BSP also showed an asymmetric orientation of densely packed chloroplasts which is consistent with their *in vivo* position. The differences in

[3] Abbreviations: WSP, water storage protoplast; MP, mesophyll protoplast; BSP, bundle sheath protoplast; RuBP, ribulose 1,5-bisphosphate; PEP, phosphoenolpyruvate; PGA, phosphoglyceric acid.

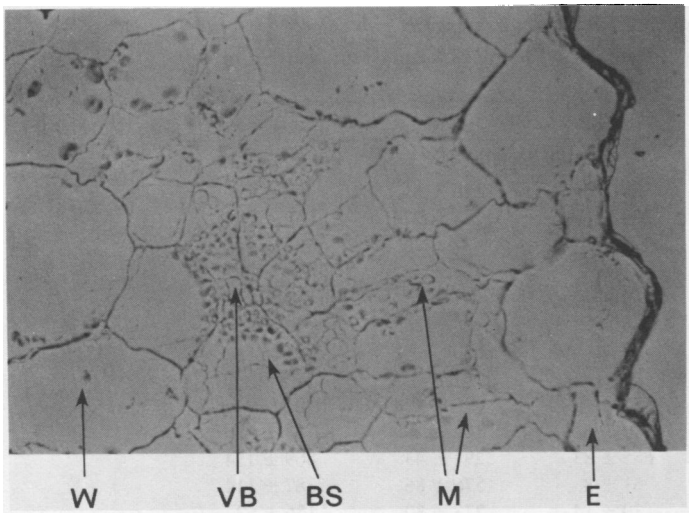

FIG. 1. Cross section of *P. grandiflora* leaf (magnification ×263). E: epidermis; M: mesophyll cell; BS: bundle sheath cell; W: water tissue; VB: vascular bundle.

both size and density of these two types of protoplasts allowed their separation according to the techniques developed by Edwards *et al.* (6). The WSP are much larger than MP and BSP, contain a large vacuole, and have only a few chloroplasts.

Under light microscopy the purity of the MP and WSP preparations was estimated to be higher than 95%. The BSP preparations were usually contaminated with 5–10% of MP.

Intercellular Localization of Enzymes. Table I shows activities of various photosynthetic enzymes in the extracts of *P. grandiflora* leaf tissues on a Chl basis. Like most C_4 plants, *P. grandiflora* has RuBP carboxylase and a C_4-decarboxylating enzyme(s), $NADP^+$-malic enzyme and NAD^+-malic enzyme, compartmentalized in the BSP. Consistent with an earlier report (7), $NADP^+$-malic enzyme is the primary decarboxylating enzyme in this species. No activity of PEP carboxykinase, another C_4-decarboxylating enzyme, was detected in the extracts of whole leaf or of the isolated cell types. Surprisingly, PEP carboxylase and pyruvate, Pi dikinase were present in all three cell types although the activity was highest in MP. The high activity of these two enzymes in the BSP extracts can not be attributed to cross contamination of 5–10% of MP in the preparations. These results suggest that bundle sheath cells and water storage cells of *P. grandiflora* are also capable of pyruvate conversion to PEP and carboxylation through PEP carboxylase. Other enzymes such as 3-PGA kinase, $NADP^+$-triose-P dehydrogenase, malate dehydrogenases ($NADP^+$ and NAD^+-dependent), alanine aminotransferase, aspartate aminotransferase, Cyt *c* oxidase, and catalase were equally distributed between MP and BSP. On a Chl basis, the enzyme profile of the WSP was similar to that of the MP but with reduced activity in most enzymes assayed, except mitochondrion-associated enzymes such as malate dehydrogenase ($NADP^+$ and NAD^+-dependent) and Cyt *c* oxidase. The Chl *a*/*b* ratios were about the same for the MP and BSP but lower for the WSP. These results are consistent with the intercellular compartmentation of photosynthetic enzymes in other C_4 plants (9, 16), except for the presence of substantial activity of PEP carboxylase and pyruvate, Pi dikinase in the bundle sheath cells of *P. grandiflora.* The equal distribution of $NADP^+$-malate dehydrogenase between MP and BSP in this species also is in contrast to the preferential compartmentation of this enzyme in mesophyll cells of other C_4 plants which contain $NADP^+$-malic enzyme as the primary decarboxylating enzyme (16).

Intracellular Localization of Enzymes. For the study of intracellular localization of enzymes, purified protoplasts were mechanically ruptured and subcellular organelles separated by differential centrifugation. Table II shows the intracellular localization of photosynthetic enzymes in the MP. The mesophyll chloroplasts in the 300*g* pellet fraction were about 75% intact as judged by retention of the chloroplast marker enzyme $NADP^+$-triose-P dehydrogenase. Among the enzymes predominantly associated with the chloroplastic fractions were pyruvate, Pi dikinase, aspartate aminotransferase, 3-PGA kinase, and $NADP^+$-malate dehydrogenase whereas PEP carboxylase was primarily in the cytosolic fraction. Alanine aminotransferase and NAD^+-malate dehydrogenase were largely cytosolic but some activities were found in the mitochondrial fraction as indicated by Cyt *c* oxidase activity.

The intracellular localization of photosynthetic enzymes in the BSP is shown in Table III. The bundle sheath chloroplasts were about 63% intact based on retention of $NADP^+$-triose-P dehydrogenase activity in the 300*g* pellet. Likewise, 64% of the RuBP

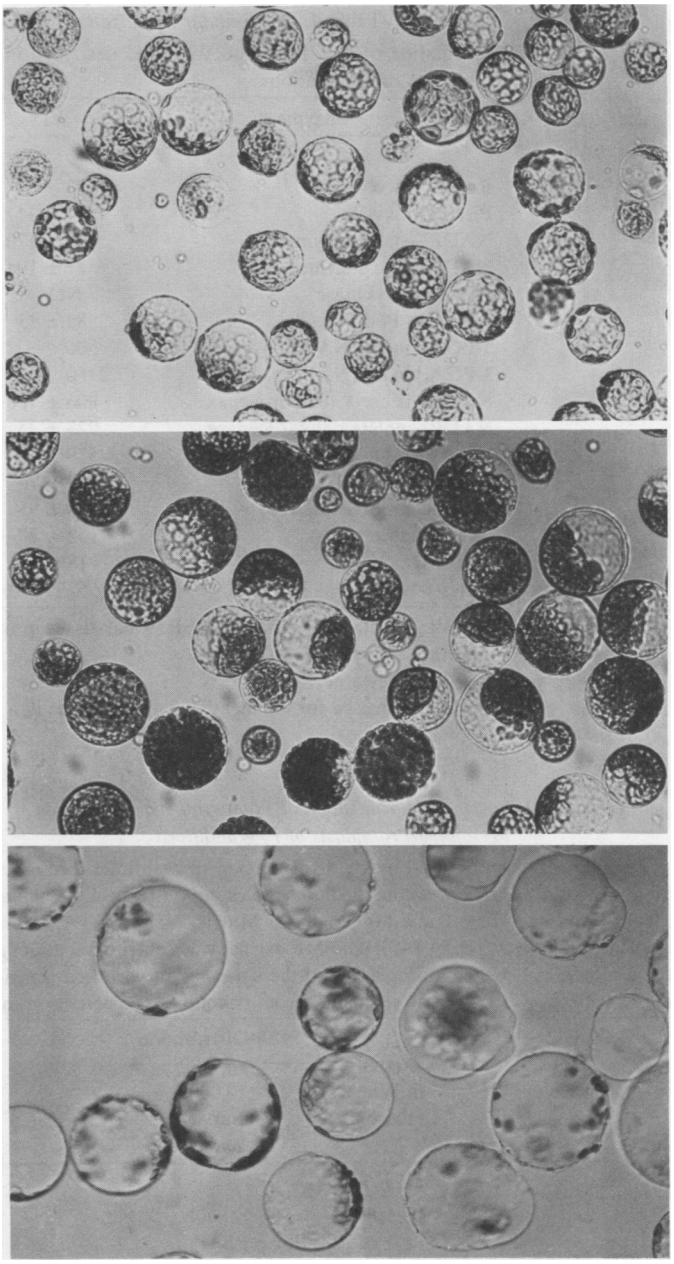

FIG. 2. Light micrographs of a field of isolated mesophyll protoplasts (*upper*) bundle sheath protoplasts (*middle*), and water tissue protoplasts (*lower*) of *P. grandiflora* (magnification ×248).

Table I. *Intercellular Localization of Photosynthetic Enzymes in Leaves of P. grandiflora*

Isolation of MP, BSP, and WSP was described in the "Materials and Methods." Error represents SD of 2–5 replicates of preparations.

Enzyme	WL[a]	WSP	MP	BSP
		μmol/mg Chl·h		
RuBP carboxylase	243 ± 51	ND[b]	19 ± 6	448 ± 22
PEP carboxylase	1537 ± 317	223 ± 67	1271 ± 276	1052 ± 356
$NADP^+$-malic enzyme	702 ± 170	29 ± 8	37 ± 28	1337 ± 465
NAD^+-malic enzyme	87 ± 19	11 ± 4	9 ± 3	120 ± 28
PEP carboxykinase	ND	ND	ND	ND
Pyruvate, Pi dikinase	82 ± 13	28 ± 5	78 ± 26	31 ± 11
3-PGA kinase ($NADP^{+c}$)	500	63	660	521
3-PGA kinase (NAD^{+c})	2180	90	2247	1745
$NADP^+$-triose-P dehydrogenase	906 ± 188	31 ± 10	698 ± 208	702 ± 94
$NADP^+$-malate dehydrogenase	223 ± 35	510 ± 61	102 ± 28	118 ± 12
NAD^+-malate dehydrogenase	579 ± 89	685 ± 83	291 ± 43	809 ± 134
Aspartate aminotransferase	447 ± 74	61 ± 8	576 ± 86	387 ± 114
Alanine aminotransferase	354 ± 95	30 ± 14	273 ± 80	156 ± 62
Cyt *c* oxidase	132 ± 20	120 ± 22	213 ± 26	145 ± 11
Catalase ($\times 10^{-3}$)	18 ± 3	7 ± 1	30 ± 6	26 ± 8
Chl *a*/*b* ratio	2.52 ± 0.26	1.72 ± 0.09	2.42 ± 0.27	2.47 ± 0.35

[a] WL, whole leaf extracts; WSP, water tissue protoplast extracts; MP, mesophyll protoplast extracts; BSP, bundle sheath protoplast extracts.

[b] Not detectable.

[c] Cofactor used for the assay of 3-PGA kinase in a reaction coupled to triose-P dehydrogenase.

Table II. *Intracellular Localization of Photosynthetic Enzymes in Mesophyll Protoplasts of P. grandiflora*

Purified MP was ruptured by gentle dispersion several times through a 50-μl microsyringe in a breaking medium containing 0.3 M sorbitol, 50 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM cysteine, and 1% (w/v) PVP-40. The MP extract was centrifuged at 300*g* for 3 min to pellet the chloroplasts and the supernatant was then centrifuged at 10,000*g* for 10 min to sediment the mitochondria and peroxisomes.

Enzyme	Activity in Whole Extract	% Distribution			% Recovery
		300*g* Pellet	10,000*g* Supernatant	10,000*g* Pellet	
	μmol/mg Chl·h				
Chl		96	ND[a]	4	104
$NADP^+$-triose-P dehydrogenase	899	75	23	2	94
Pyruvate, Pi dikinase	78	60	21	19	112
Aspartate aminotransferase	514	66	29	6	99
3-PGA kinase ($NADP^{+b}$)	660	56	40	4	94
3-PGA kinase (NAD^{+b})	2247	64	32	4	131
$NADP^+$-malate dehydrogenase	102	69	27	4	89
PEP carboxylase	1241	10	84	6	108
Alanine aminotransferase	330	11	77	12	87
NAD^+-malate dehydrogenase	291	26	56	18	95
Cyt *c* oxidase	139	20	1	79	108
Catalase ($\times 10^{-3}$)	26	13	54	33	134

[a] Not detectable.

[b] Cofactor used for the assay of 3-PGA kinase in a reaction coupled to triose-P dehydrogenase.

carboxylase activity and 67% of the $NADP^+$-malic enzyme activity were associated with the chloroplast fraction indicating that they are chloroplast enzymes. Also, among the enzymes predominantly associated with the chloroplast fraction were pyruvate, Pi dikinase, aspartate aminotransferase, 3-PGA kinase, and $NADP^+$-malate dehydrogenase. Similar to the MP, the PEP carboxylase activity associated with the BSP was cytosolic. Alanine aminotransferase and NAD^+-malate dehydrogenase were found in both cytosolic and mitochondrial fractions indicating that these two enzymes are present in both the cytosol and the mitochondria.

The above experiments were repeated several times and the results were always consistent although the actual values were variable from experiment to experiment. No attempt was made to isolate subcellular organelles from the WSP since most of the enzymes examined in this tissue were low in activity.

These results on intracellular localization of photosynthetic enzymes are in good agreement with results obtained previously with two other C_4 plants, MP of maize (8) and BSP of *Panicum miliaceum* (6).

Diurnal Fluctuation of Titratable Acidity and Malate Concentration. Recent studies suggest that succulent C_4 dicots may be capable of developing CAM under certain conditions (13, 14). To evaluate the maximal diurnal acid fluctuations in *P. grandiflora*, total titratable acidity and malate concentration were measured with leaf and stem tissues sampled at the end and the beginning of the photoperiod. Well-watered plants of *P. grandiflora* showed significant diurnal fluctuations of acidity in both leaf and stem tissues, with an amplitude of 61 and 54 μeq/g fresh weight for the leaves and stems, respectively (Table IV). The changes in titratable acidity in these tissues were in parallel with changes in malic acid concentration although the changes in malic acid were not enough to account for the total changes in acidity. These results suggest that a considerable amount of CO_2 was fixed by the plants during the dark period (acidification), presumably via PEP carboxylase, and that deacidification occurred during the light period through C_4-decarboxylating mechanisms. Under severe drought conditions, diurnal changes in both titratable acidity and malate concentration in leaves and stems of *P. grandiflora* were much reduced. These results with the succulent C_4 dicot *P. grandiflora* are in contrast to those obtained with *P. afra* (Talbe IV), an inducible

Table III. *Intracellular Localization of Photosynthetic Enzymes in Bundle Sheath Protoplasts of P. grandiflora*

Purified BSP was ruptured by gentle dispersion several times through a 50-μl microsyringe in a breaking medium containing 0.3 M sorbitol, 50 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM cysteine, and 1% (w/v) PVP-40. The BSP extract was centrifuged at 300*g* for 3 min to pellet the chloroplasts and the supernatant was then centrifuged at 10,000*g* for 10 min to sediment the mitochondria and peroxisomes.

Enzyme	Activity in Whole Extract	% Distribution			% Recovery
		300*g* Pellet	10,000*g* Supernatant	10,000*g* Pellet	
	μmol/mg Chl·h				
Chl		93	ND[a]	7	100
RuBP carboxylase	443	64	35	1	83
$NADP^+$-triose-P dehydrogenase	597	63	35	2	87
$NADP^+$-malic enzyme	1022	67	30	3	97
Pyruvate, Pi dikinase	31	58	32	10	105
Aspartate aminotransferase	517	57	39	4	94
$NADP^+$-malate dehydrogenase	117	59	38	3	107
3-PGA kinase ($NADP^{+b}$)	521	62	36	2	83
3-PGA kinase (NAD^{+b})	1745	52	46	2	104
PEP carboxylase	1519	3	95	2	73
Alanine aminotransferase	152	9	77	15	110
NAD^+-malate dehydrogenase	822	3	71	26	96
Cyt *c* oxidase	148	29	25	47	111
Catalase ($\times 10^{-3}$)	32	16	31	53	96

[a] Not detectable.

[b] Cofactor used for the assay of 3-PGA kinase in a reaction coupled to triose-P dehydrogenase.

CAM plant (23). Well-watered plants of *P. afra* exhibited little or no diurnal fluctuation of acidity and malate concentration in both leaf and stem tissues despite having very high levels of acids. When *P. afra* plants were subjected to severe water stress, however, the diurnal change of titratable acidity in the leaves was amplified to 182 μeq/g fresh weight, which was paralleled by the changes in malate concentration. Water status did not affect the total acid fluctuations in stems, but malate concentrations of the water-stressed stems were 4- to 5-fold higher than those of the control stems.

It is not certain if deacidification-acidification in the day-night cycle is a general characteristic of C_4 dicots. A nonsucculent C_4 dicot *Amaranthus graecizans* was included in the study for comparison (Table IV). Under either well-watered or water-stressed conditions, this plant did not have significant diurnal fluctuations of titratable acidity and malate concentration in leaves and stems although the acid levels in these tissues were comparable to those of *P. grandiflora*. Koch and Kennedy (14) reported that *P. oleracea*, another succulent C_4 dicot, exhibits a CAM-like pattern of acid fluctuation in both leaves and stems when grown under short-day and well-watered conditions. Subsequently, Karadge and Joshi (13) found that the CAM-like activity of this plant is due to the synthesis and accumulation of organic acids (mainly malate) during the night. The amplitude of acid fluctuation in *P. oleracea* was reduced in water-stressed plants (14), similar to the response of *P. grandiflora*. The effect of photoperiod on acid metabolism in *P. grandiflora* was not examined in this study. It appears that some succulent C_4 dicots, but not all C_4 dicots, are capable of CAM under certain environmental conditions.

DISCUSSION

The C_4 dicot *P. grandiflora* has cylindrical, succulent leaves with net veination similar to a dicot. The peripherally arranged bundle sheaths are well-developed and contain numerous chloroplasts in a centripetal position. Mesophyll cells occur only in the area between the bundle sheath and the epidermis rather than forming a complete layer surrounding the bundle sheath tissue. This characteristic is in contrast to the common type of Kranz leaf anatomy which consists of a layer of mesophyll cells completely surrounding the bundle sheath. In addition to these two cell types common to other C_4 plants, *P. grandiflora* possesses a third distinct type of green cell, water storage cells, located in the center of the leaves. Somewhat similar variations in Kranz leaf anatomy have been reported in two other succulent C_4 dicots, *Salsola kali* (19, 25), and *Suaeda monoica* (21). The cylindrical leaves of these two species have an outer layer of palisade cells and an inner layer of chlorenchyma sheath cells which surround the water tissue. The biochemical functions of the palisade cells are similar to those of mesophyll cells and the inner chlorenchyma sheath is similar to the bundle sheath cells in a typical C_4 plant (19, 22). The specialized leaf anatomy of these succulent species may have an adaptive advantage. These species are known to grow in an open strand community with xeric and high light conditions. The cylindrical leaves have a large volume of water tissue and a low surface area to volume ratio which aids water retention. In this respect they are very similar to CAM plants. Unlike CAM plants, however, the cylindrical leaves of these species are capable of efficient CO_2 fixation because of differentiation of the chlorenchyma into essentially mesophyll and bundle sheath cells. Mesophyll cells of these C_4 dicots having cyclindrical, succulent leaves are advantageously located in the area between the bundle sheath tissue and the epidermis where atmospheric CO_2 is most accessible. Thus, the site of initial CO_2 fixation is likely in these mesophyll cells whose anatomical location is typical of C_4 plants.

P. grandiflora may have a slightly different pathway of carbon assimilation than other C_4 plants as suggested by the results on inter- and intracellular localization of key photosynthetic enzymes in their leaves (Tables I, II, III). Similar to other C_4 plants, RuBP carboxylase of the C_3 pathway and the decarboxylating enzyme $NADP^+$-malic enzyme are compartmentalized only in the bundle sheath cells. However, unlike other C_4 plants, PEP carboxylase and pyruvate, Pi dikinase of the C_4 pathway are present in all three cell types suggesting that all are capable of fixing CO_2 via PEP carboxylase and regenerating PEP from pyruvate. The contribution of the water tissue to net photosynthesis in this species may be limited since the Chl content and the activity of photosynthetic enzymes on a Chl basis are low in addition to the long physical diffusion pathway of CO_2 from the atmosphere. However, the water storage tissue appears to be a likely site for fixation of internally released CO_2 in this succulent tissue particularly at night.

Based on the data of enzyme localization presented in this study, a scheme of C_4 photosynthesis in *P. grandiflora* is proposed as shown in Figure 3. This scheme is consistent with the concept of a division of labor between photosynthetic cells with malate and pyruvate as the primary transport metabolites between mesophyll and bundle sheath cells in the C_4 shuttle system. Because of the anatomical arrangement of mesophyll cells, most of the atmospheric CO_2 would be fixed initially through PEP carboxylase in the cytosol of mesophyll cells into oxalacetate, although a minor portion of atmospheric CO_2 could be directly transported to and fixed in bundle sheath cells or water storage cells through PEP carboxylase. The oxalacetate formed is then reduced to malate

Table IV. *Titratable Acidity and Malate Concentration in Leaves and Stems for Control and Water-Stressed Plants of P. grandiflora, A. graecizans, and P. afra*

Leaf and stem samples were removed from the plants at the end and the beginning of the photoperiod.

Species	Treatment	Tissue	Water Content	Titratable Acidity			Malic Acid		
				10:30 PM	7:30 AM	Δ	10:30 PM	7:30 AM	Δ
			g H_2O/g dry wt	*μeq/g fresh wt*			*μmol/g fresh wt*[a]		
P. grandiflora	Control	Leaf	14.0	15	76	61	3.2	11.9	8.7
		Stem	9.0	27	81	54	1.9	7.2	5.3
	Water-stressed	Leaf	9.3	11	45	34	1.4	2.8	1.4
		Stem	8.7	28	42	14	1.0	3.3	2.3
A. graecizans	Control	Leaf	7.0	76	82	6	0.3	0.5	0.2
		Stem	9.1	37	43	6	1.5	1.5	0.0
	Water-stressed	Leaf	5.5	81	90	9	0.8	1.3	0.5
		Stem	9.0	42	47	5	2.2	2.3	0.1
P. afra	Control	Leaf	16.5	233	248	15	25.7	25.8	0.1
		Stem	7.2	112	147	35	5.1	8.3	3.2
	Water-stressed	Leaf	8.2	138	320	182	18.8	61.1	42.3
		Stem	5.3	76	119	43	24.6	32.8	8.2

[a] One μmol of malic acid is equivalent to 2 μeq of acidity.

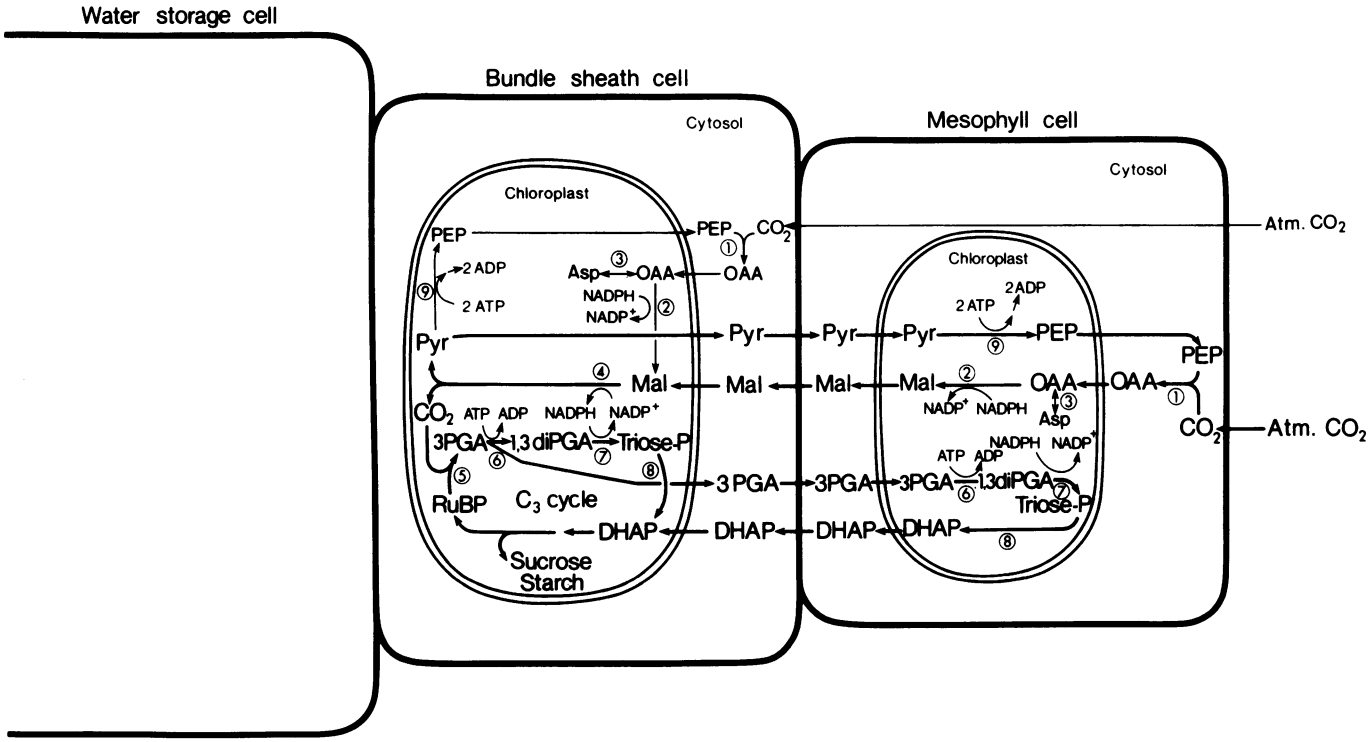

FIG. 3. A proposed scheme of C_4 photosynthesis in *P. grandiflora*. (1) PEP carboxylase, (2) $NADP^+$-malate dehydrogenase, (3) aspartate aminotransferase, (4) $NADP^+$-malic enzyme, (5) RuBP carboxylase, (6) 3-PGA kinase, (7) trisoe-P dehydrogenase, (8) triose-P isomerase, and (9) pyruvate, Pi dikinase.

catalyzed by $NADP^+$-malate dehydrogenase or converted to aspartate by aspartate aminotransferase in the mesophyll chloroplasts. Consistent with $NADP^+$-malic enzyme as the primary decarboxylating mechanism in this species, malate instead of aspartate is proposed to be the major photosynthetic metabolite transported across mesophyll cells to bundle sheath cells, presumably through plasmodesmata connecting the two cell types (20). Malate moves into the bundle sheath chloroplasts and is decarboxylated via $NADP^+$-malic enzyme releasing CO_2 and pyruvate. Thus malate serves as a CO_2 carrier from mesophyll to bundle sheath cells. The released CO_2 is then reassimilated in the C_3 cycle of photosynthesis to the level of carbohydrate. For the regeneration of the C_3 precursor PEP, pyruvate is shuttled back to the mesophyll chloroplasts where pyruvate, Pi dikinase converts it to PEP.

Some activity of pyruvate, Pi dikinase was associated with the bundle sheath chloroplasts of *P. grandiflora* (Tables I, III). In a previous study, Ku and Edwards (16) also reported substantial

activity of pyruvate, Pi dikinase in the bundle sheath cells of two other $NADP^+$-malic enzyme type C_4 plants, *Pennisetum purpureum* and *Zea mays*. However, very little activity was found in the bundle sheath cells of NAD^+-malic enzyme and PEP carboxykinase type C_4 plants. $NADP^+$-malic enzyme type C_4 plants are known to have normal PSI but deficient PSII activity in bundle sheath cells (4, 15). We speculated that part of the pyruvate released in the bundle sheath chloroplasts may be converted to PEP in the same organelle using excess ATP produced by PSI in these species. Whether the resulting PEP formed in the bundle sheath chloroplasts would be transported back to mesophyll cells is uncertain but in *P. grandiflora* this is no problem since PEP carboxylase is present in bundle sheath cells. PEP carboxylation in bundle sheath cells may be minimal during the day due to lack of excess CO_2 from atmosphere or chloroplasts. However, during the dark respiratory CO_2 will be trapped in bundle sheath cells by PEP carboxylase (acidification).

Energy in the form of ATP and NADPH is required in the C_4 as well as in the C_3 pathway of photosynthesis. In $NADP^+$-malic enzyme type C_4 plants such as *P. grandiflora*, two ATP and one NADPH are required for each malate formed in the mesophyll chloroplasts. Along with the decarboxylation of malate in the bundle sheath chloroplasts by $NADP^+$-malic enzyme, energy in the form of one NADPH per malate would be transported from mesophyll to bundle sheath cells. However, for each CO_2 donated to the C_3 cycle through malate decarboxylation two ATP and two NADPH are required for the reduction of 3-PGA to the level of triose phosphate and one additional ATP for the regeneration of RuBP. The transfer of one NADPH per malate from mesophyll to bundle sheath cells only partially satisfies this requirement. Bundle sheath cells of $NADP^+$-malic enzyme type C_4 species have been shown to have a reduced capacity for PSII, *i.e.* transferring electrons from water to $NADP^+$ (11, 15). However, in *P. grandiflora* the capacity of PSI and PSII appears to be similar in mesophyll and bundle sheath chloroplasts based on the equal Chl *a/b* ratios between cell types (Table I). Therefore, depending on the species there may be as much as 50% of the 3-PGA produced in the bundle sheath cells transported back to the mesophyll cells for reduction. In *P. grandiflora*, the percentage of 3-PGA reduction between cell types is unknown. Mesophyll chloroplasts lack RuBP kinase and RuBP carboxylase but contain the enzyme complement for the reductive phase of the C_3 cycle. Hatch and colleagues (4, 11) presented data indicating that the partitioning of 3-PGA reduction between cells occurs not only in the $NADP^+$-malic enzyme species but also in species from other groups. Dihydroxyacetone phosphate is most likely the metabolite transported back to the bundle sheath chloroplasts based on permeability studies of C_3 chloroplasts to photosynthetic metabolites (2).

Koch and Kennedy (14) reported a CAM-like acid metabolism in a succulent C_4 dicot *P. oleracea* when grown under short day and well-watered conditions. The CAM-like activity of this plant is mainly due to the synthesis and accumulation of malate during the night (13). In the present study, with another succulent C_4 dicot from the same genus *P. grandiflora* we also observed a similar day/night fluctuation of total acidity and malate concentration in both leaves and stems. Our results thus confirm the suggestion by Koch and Kennedy that succulent C_4 dicots may be capable of acidification-deacidification similar to that of CAM plants under certain environmental conditions. Our data also suggest that the capability of acid metabolism is not a general characteristic of all C_4 dicots. Only succulent C_4 dicots are capable of acid metabolism. The capacity for acid metabolism may be dependent on species as well as on environmental conditions. Effects of endogenous and various environmental factors such as developmental stage, photoperiod, temperature, and light intensity on acid metabolism and gas exchange pattern in *P. grandiflora* awaits further investigation.

Our acid fluctuation studies suggest that PEP carboxylase is involved in the noctural acidification. Respiratory CO_2 in these thick, succulent leaf and stem tissues is most likely refixed in the dark via PEP carboxylase although atmospheric CO_2 as a source can not be ruled out. As to the carbon source for the synthesis of the C_3 precursor PEP in the dark, it is difficult to visualize since the conversion of pyruvate to PEP in the C_4 chloroplasts requires ATP derived from the light reactions. A glycolytic pathway similar to that in CAM plants, leading from glucan to PEP for malate synthesis, may be operating in these succulent species but this too awaits investigation.

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