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Short Communication

Pyruvate, Pi Dikinase in Bundle Sheath Strands as Well as in Mesophyll Cells in Maize Leaves[1]

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ABSTRACT

Mesophyll protoplasts and bundle sheath strands were isolated from maize leaves. Light microscopic observation showed the preparations were pure and without cross contamination. Protein blot analysis of mesophyll and bundle sheath cell soluble protein showed that the concentration of pyruvate orthophosphate dikinase (EC 2.7.9.1) is about one-tenth as much in the bundle sheath cells as in mesophyll cells, but about eight times greater than that found in wheat leaves, on the basis of soluble protein. Phosphoenolpyruvate carboxylase (EC 4.1.1.31) was barely detectable in the bundle sheath cells, while ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) and NADP-dependent malic enzyme (EC 1.3.1.37) were exclusively present in the bundle sheath cells and were absent in the mesophyll cells. Whereas pyruvate, Pi dikinase was previously considered localized only in mesophyll cells of C_4 plants, these results clearly demonstrate the presence of appreciable quantities of the enzyme in the bundle sheath cells of the C_4 species maize.

The C_4 pathway is characterized by the initial assimilation of CO_2 and C_4 acids in mesophyll cells and subsequent decarboxylation and reduction of the carbon by the RPP[2] pathway in the bundle sheath cells (4, 7). Previous studies have provided evidence for the differential localization of certain enzymes involved in the C_4 pathway (7). For example, PEPC, PPDK, and NADP-dependent MDH are predominantly present in mesophyll cells and RuBPC and NADP-dependent ME are predominantly present in bundle sheath cells.

Though data obtained in various laboratories have supported this differential localization of certain enzymes, the results may not be conclusive since most of the data were obtained by measurements on enzyme activity only. If extraction and/or assay conditions are not optimal, potentially low levels of enzyme activity may go undetected. Furthermore, some enzymes have been shown to be present in inactive forms (6). PPDK and NADP-dependent MDH are known to be activated by illumination in C_4 plants (10, 14). Immunochemical methods have been successfully applied to localize a few enzymes in C_4 plants, namely RuBPC, PEPC, and NADP-dependent MDH (20, 22, 23). These methods have the advantage of high sensitivity and reactivity even to an inactive form (*i.e.* NADP-dependent MDH) (23).

Aoyagi and Bassham (1) have demonstrated the presence of PPDK in leaves and seeds of C_3 plants using a sensitive immunochemical method. The PPDK activity of C_3 leaves is only 1 to 5% of that found in C_4 maize leaves so that detection by the activity assay can be difficult (19, 28).

PPDK is thought to be a rate-limiting enzyme for C_4 photosynthesis (10, 26, 27). There have been no immunochemical studies on its intercellular localization. Since PPDK is somewhat unstable, light-activated, and present in relatively low activity, immunochemical methods may be a more reliable means to determine its localization. A low activity of PPDK was detected in bundle sheath strands from maize but not from other C_4 plants (H. Nakamoto, unpublished data). The instability of the enzyme combined with its low activity in these preparations made it difficult to obtain reproducible results.

We have now investigated immunochemically the differential localization of PPDK, PEPC, RuBPC, and NADP-dependent ME using mesophyll protoplasts and bundle sheath strands from maize leaves, and we find evidence for the presence of appreciable amounts of PPDK in bundle sheath strands of this species.

MATERIALS AND METHODS

Plant Material. Maize (*Zea mays* L. cv Golden Bantam) was grown in vermiculite in a growth chamber with a quantum flux density of 600 μE m^{-2} s^{-1} (400–700 nm) with an 18-h photoperiod at 27°C. Wheat (*Triticum aestivum* L. cv Anza) was grown with a quantum flux density of 250 μE m^{-2} s^{-1} (400–700 nm) with a photoperiod of 8 h and at 15°C. The wheat seeds were a gift from Dr. Calvin Qualset, University of California, Davis. The plants were watered every other day with modified half-strength Hoagland solution. The maize leaves (2–3 weeks old, third leaf) were harvested and used immediately. The part of the wheat leaf used was the tip of the sixth leaf (7 weeks after planting). The top 4 cm were cut from 42-cm long leaf blades and were frozen in liquid N_2 and stored at −80°C until used.

Reagents. Pectinase (macerozyme R-10) and cellulase R-10 were obtained from Yakult Biochemical Co., Ltd., Nishinomiya, Japan. I^{125} protein A (30 mCi/mg) was purchased from Amersham Co. Other reagents were purchased from either Sigma Chemical Co. or Biorad Laboratories.

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[2] *Abbreviations*: RPP, reductive pentose phosphate; ME, malic enzyme; PPDK, pyruvate orthophosphate dikinase; PEPC, phosphoenolpyruvate carboxylase; RuBPC, ribulose 1,5-bisphosphate carboxylase; MDH, malate dehydrogenase; PMSF, phenylmethylsulfonyl fluoride.

Isolation of Bundle Sheath Strands and Mesophyll Cell Protoplasts. Isolation was carried out after modifications of previous methods (8, 18). Seven g of maize leaves were cut into 0.5 mm segments or less with a razor blade. The segments were placed in 50 ml of digestion medium consisting of 4% Onozuka R-10 cellulase, 0.2% macerozyme R-10, 0.1% BSA, 1 mM $CaCl_2$, 0.5 M sorbitol, and 10 mM Mes-KOH buffer (pH 5.5). Incubation was carried at 27°C with a quantum flux density of 600 $\mu E\ m^{-2}\ s^{-1}$ (400–700 nm) for 2.5 h. After the completion of digestion was confirmed by light microscopy, the digestion medium was removed by a Pasteur pipet. The tissues were washed four times in 5 ml of solution A containing 0.5 M sucrose, 1 mM $CaCl_2$, and 5 mM Hepes-KOH (pH 7.0).

The solution was filtered through a 500-μm nylon net and then an 80-μm net. Bundle sheath strands remained on the 80 μm net. They were washed four times with 7 ml of solution A. At this stage, light microscopic observation showed the bundle sheath preparation to be free of mesophyll cells (Fig. 1).

Mesophyll protoplasts which were in the filtrate were overlaid with solution B consisting of 0.5 M sorbitol, 1 mM $CaCl_2$, and 5 mM Hepes-KOH (pH 7.0) and centrifuged at 100 g for 5 min. Purified mesophyll protoplasts were diluted with solution B and centrifuged at 100 g for 2 min. The protoplast pellet was resuspended in 0.3 ml of solution B and kept on ice. The light microscopic observation showed intact protoplasts free of bundle sheath cells.

Protein Blot. Bundle sheath strands were homogenized in 1 ml of 0.1 M Tris buffer (pH 7.4) containing 10 mM $MgCl_2$, 18% w/v sucrose, 1% β-mercaptoethanol, and 10 mM PMSF using mortar and pestle. Mesophyll cell protoplasts suspended in 0.3 ml of solution B were homogenized in a glass homogenizer with 0.6 ml of the above buffer. Whole maize leaves were homogenized with 3 volumes of the same buffer. The homogenate was filtered through Miracloth and then centrifuged at 12,000 g for 10 min. The total soluble protein was determined by the method of Bradford (5). The Chl *a*/Chl *b* ratio was determined according to Arnon (3).

Varying quantities of soluble protein (20–60 g) from bundle sheath strands, mesophyll protoplasts, and whole leaves were loaded for a comparison on polyacrylamide gradient gels (6.4–12.8%). After SDS-PAGE, the protein was transferred to cyanogen bromide paper at 0.5 amp for 9 h. After transfer, the gel was stained with Coomassie brilliant blue and destained to check the transfer efficiency. The transfer paper was probed with anti-PPDK serum or anti-PEPC serum, then with 0.5 μCi of I^{125} protein A. Autoradiographs were prepared using Kodak AR5 x-ray film with intensifying screens at −80°C overnight. After the film was developed, the paper was again probed with either anti-RuBPC or anti-NADP dependent ME sera. Antisera to maize RuBPC and PEPC were kindly provided by Dr. William Taylor, UC, Berkeley (21). Antiserum to maize NADP-dependent ME was kindly provided by Dr. Timothy Nelson, Yale University. Antiserum to maize PPDK was prepared as described previously (1).

The amount of each enzyme polypeptide was estimated by densitometry of the x-ray film as described previously (1).

Light Microscopy. The mesophyll protoplasts and bundle sheath strands, suspended in solution B and A, respectively, were examined with a Nikon phase contrast microscope equipped with a Nikon polaroid camera. Both mesophyll protoplasts and bundle sheath strands remained intact at 4°C for several days.

RESULTS

Preparations of mesophyll cell protoplasts were free from contamination by bundle sheath cells according to light microscopic observation. Bundle sheath strands preparations were free from mesophyll cell contamination (Fig. 1). Since the bundle sheath strands were carefully washed four times with 7 ml of the buffer, contamination by stromal enzymes from broken mesophyll cell chloroplasts should be negligible. The Chl *a*/Chl *b* ratio of bundle sheath cells was 9 to 10, whereas that of mesophyll cells was 3.2. This difference in Chl *a*/Chl *b* ratios of the two preparations is consistent with the expected values for the purified cell types (11, 18).

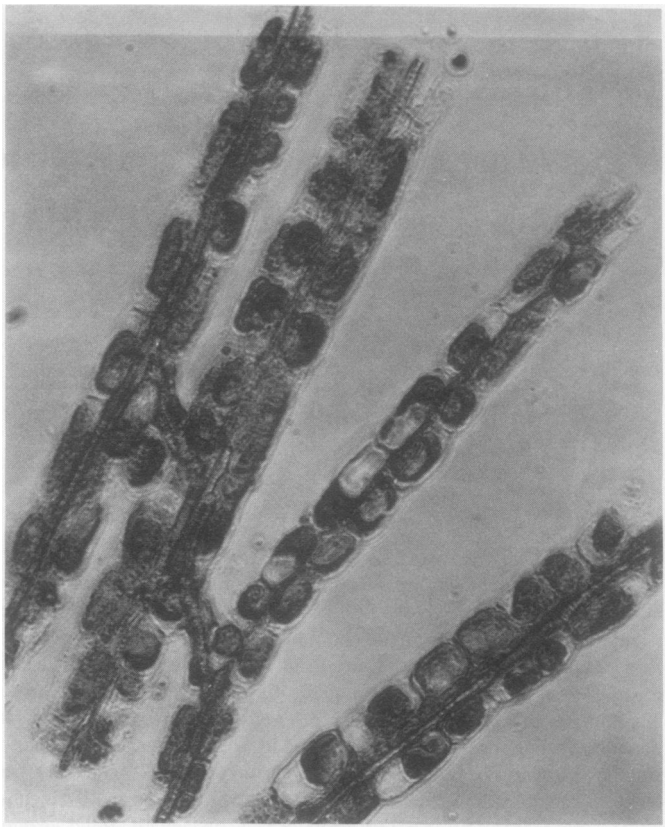

FIG. 1. Light microscopy of bundle sheath strands of maize, ×80.

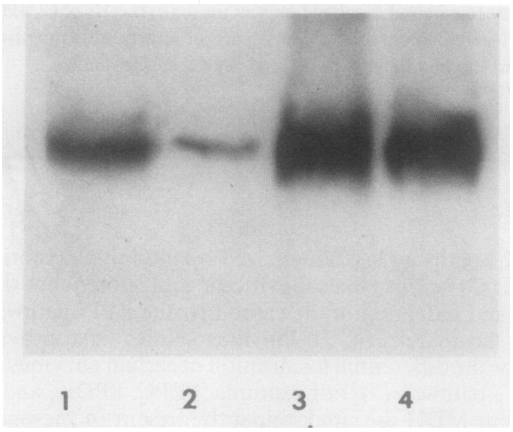

FIG. 2. Protein blot analysis for PPDK of various samples. Lane 1, Wheat leaf tip (400 μg soluble protein); lane 2, bundle sheath strands of maize (20 μg soluble protein); lane 3, mesophyll cells of maize (20 μg soluble protein); lane 4, whole leaf of maize (20 μg soluble protein).

According to protein blot analysis of the soluble protein for PPDK, the enzyme is present in bundle sheath cells at approximately one-tenth of the concentration found in the mesophyll cells on soluble protein basis (Fig. 2). The amount of PPDK in maize bundle sheath strands is higher than the highest level we have found so far in wheat leaves. Only a minute amount of

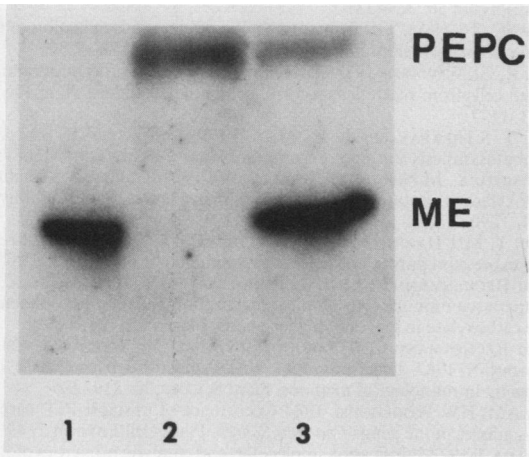

FIG. 3. Protein blot analysis for PEPC and NADP-dependent ME from maize leaves. Lane 1, Bundle sheath strands (60 μg soluble protein); lane 2, mesophyll cells (60 μg soluble protein); lane 3, whole leaf (60 μg soluble protein).

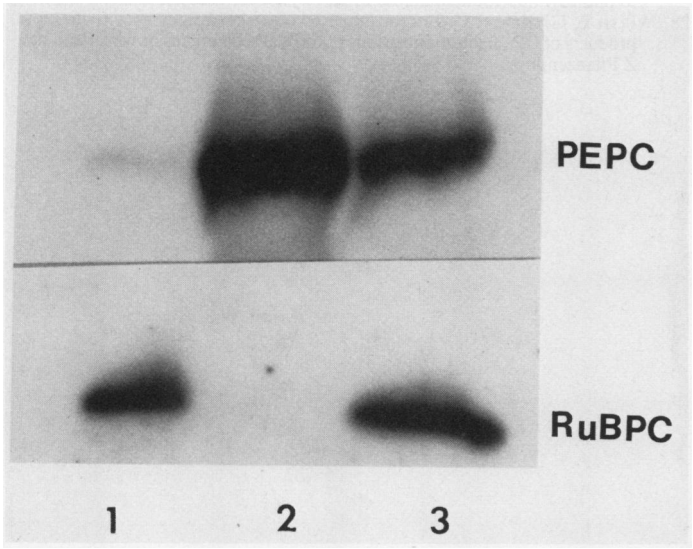

FIG. 4. Protein blot analysis for RuBPC and PEPC from maize leaves. Lane 1, Bundle sheath strands (40 μg soluble protein); lane 2, mesophyll cells (40 μg soluble protein); lane 3, whole leaf (40 μg soluble protein).

PEPC is seen in the bundle sheath strands when the blot was probed with anti-PEPC and anti-ME (Fig. 3). In fact, another experiment did not show the presence of PEPC even when 200 μg of soluble protein was loaded. ME is absent in mesophyll cells, but present in both bundle sheath strands and the whole leaf.

Probing the blot with anti-PPDK and anti-RuBPC demonstrates the presence of RuBPC in the bundle sheath strands and whole leaf and provides an indication of the good separation of the cell types without cross-contamination (Fig. 4).

DISCUSSION

Differences in enzymic activities between mesophyll cells and bundle sheath strands can provide important information on distribution of enzymes (7, 18). Electrophoresis is also useful in examining segregation of enzymes between the two cell types (13, 17). Activity assays may, however, underestimate the amount of enzyme due to inactivation during preparation (25). The use of electrophoresis will not detect modified enzymes or distinguish between enzymes when bands are too closely spaced. Protein blot, on the other hand, can detect inactivated or even degraded enzyme polypeptide present in less than 0.1 μg or at 0.05% of the total soluble protein. This is more sensitive than some other immunoassay method by more than 10-fold (20).

Our present results confirm the exclusive distribution of RuBPC in the bundle sheath strands (15, 17, 20). We also confirm by immunochemistry the exclusive distribution of NADP-dependent ME in the bundle sheath strands, as was found by previous studies with activity measurements of the enzyme obtained by various laboratories. We confirm that PEPC is predominantly located in mesophyll cells as reported earlier (22), though it is conceivable that a small amount of the enzyme is present in bundle sheath strands. Smith and Woolhouse (24), have shown the existence of some PEPC in leaves of *Spartina anglica*, a C_4 plant, from which mesophyll cells were enzymically removed. They also provided evidence from kinetic studies that the enzyme may be an isozyme. However, it is not known if the isozyme is immunochemically different from the enzyme in mesophyll cells and regulated by a different gene.

In contrast to the exclusive localization of NADP-dependent ME and RuBPC in bundle sheath strands, and the nearly exclusive location of PEPC in leaf mesophyll cells, PPDK was present not only in mesophyll cells but also occurs in bundle sheath strands of maize in substantial amounts. PPDK is coded by nuclear gene(s). When destined for chloroplasts, it is initially synthesized in the cytoplasm as a 110,000-D polypeptide (*i.e.* subunit size) which is post-translationally processed to a 94,000-D polypeptide when it is transported into the chloroplasts (12). However, Aoyagi and Bassham (2) showed that PPDK from wheat and maize seeds is not synthesized as precursor which is post-translationally processed and they further suggested that PPDK in the seed may be coded by different genes. It is not known whether PPDK in bundle sheath strands found in the present study is coded by a different gene from that in mesophyll cells or in seed tissues.

In order for C_4 photosynthesis to function most efficiently it is reasonable that certain enzymes, notably PEPC, RuBPC, and NADP-ME, have complete differential localization between the two photosynthetic cell types. The C_4 pathway acts as an intercellular CO_2 carrier (4, 9). However, some other photosynthetic enzymes, including those for reduction of glycerate I-P to glyceraldehyde 3-P function in both mesophyll and bundle sheath cells. The present study suggests that in maize some of the pyruvate generated from malate decarboxylation through NADP-ME may be converted to PEP through PPDK in bundle sheath cells. Thus, both cell types may share in the regeneration of the substrate for PEP carboxylase. In addition, if some PGA is converted to PEP in mesophyll cells (16) it would not be necessary that all of the pyruvate from malate decarboxylation return to the mesophyll cell. These possibilities add flexibility in how the level of carbon intermediates of the C_4 cycle may be maintained.

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