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Cell-Specific Expression of Pyruvate, Pi Dikinase[1]

IN SITU mRNA HYBRIDIZATION AND IMMUNOLOCALIZATION LABELING OF PROTEIN IN WHEAT SEED

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ABSTRACT

Pyruvate, Pi dikinase (PPDK) is a key enzyme in the C4 photosynthetic pathway. However, its metabolic role in C3 plants remains uncertain. Northern blot analyses of PPDK mRNAs from wheat leaves and seeds probed with maize PPDK cDNA indicates the presence of organ-specific mRNAs. Immunofluorescent labeling of protein in wheat seed demonstrate that the PPDK polypeptide and the ribulose-1, 5-bisphosphate carboxylase small subunit polypeptide are localized predominantly in the aleurone layer and the chlorophyllous pericarp tissue, respectively. This differential distribution of the two polypeptides in wheat seed is paralleled by the differential localization of the their mRNAs as revealed by *in situ* hybridization. These results suggest a distinct role of cytoplasmic PPDK in seeds, which is different from the well established role in C4 photosynthesis.

Pyruvate, Pi dikinase (PPDK: EC 2.7.9.1)[2] is an important enzyme in the photosynthetic C4 carbon transport pathway. It catalyzes the reaction of pyruvate + Pi to form PEP, the substrate of PEPC (EC.4.1.1.31). For a long time, it was believed that C3 plants lacked this enzyme. Later the presence of PPDK was reported in immature C3 seeds (9, 18), and C3 leaves (2, 15) even though its amount was much lower than that found in maize leaf.

In both wheat and maize seeds, the PPDK subunit is synthesized on cytoplasmic ribosomes as a 94 kD polypeptide (3). On the other hand, in maize leaves (3, 11) and in wheat leaves (3) it is synthesized as a 110 kD precursor containing a 16 kD transit sequence. The precursor is imported into chloroplasts and processed to give the 94 kD polypeptide (5).

Recently the expression of specific PPDK mRNAs in different maize organs was reported by Hudspeth *et al.* (13) by Northern blot analysis. The size of the leaf PPDK mRNA was 3.5 kb, while that of the root mRNA was 3.0 kb. The difference in the mRNA size of 0.5 kb corresponds to the presence or absence of a sequence encoding the transit peptide. In this study we demonstrate the organ-specific localization of mRNAs in seeds and leaves of both wheat and maize by Northern blot analysis using a maize PPDK cDNA which allowed us to estimate the sizes and relative abundance of mRNAs.

In leaves, PPDK enzyme activity is regulated by a regulatory protein factor in the light (7). In seeds, however, it is not light regulated (18). In order to elucidate the role of PPDK in developing wheat seeds, we have reexamined its localization by both *in situ* immunofluorescent labeling of the PPDK polypeptide and *in situ* mRNA hybridization. This was necessary because previous reports on the presence of PPDK in pericarp tissue have

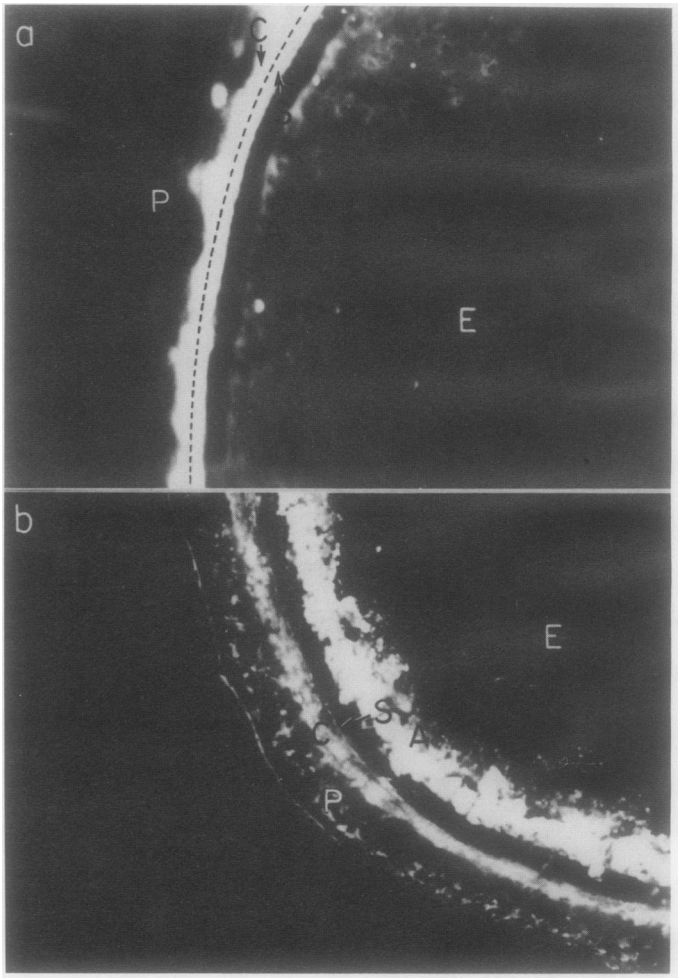

FIG. 1. Immunofluorescent labeling of PPDK and rbcS within the wheat seed. The cyrostat sections were probed with antiserum to rbcS (a), PPDK (b) or nonimmunized serum for a control. Control sections showed no specific labeling. A, Aleurone layer; C, cross cell; E, endosperm; P, pericarp; and S, seed coat.

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[2] Abbreviations: PPDK, pyruvate, Pi dikinase; RUBISCO, ribulose 1,5-bisphosphate carboxylase; rbcS, RUBISCO small subunit; PEP, phosphoenolpyruvate; PEPC, phosphoenolypyruvate carboxylase.

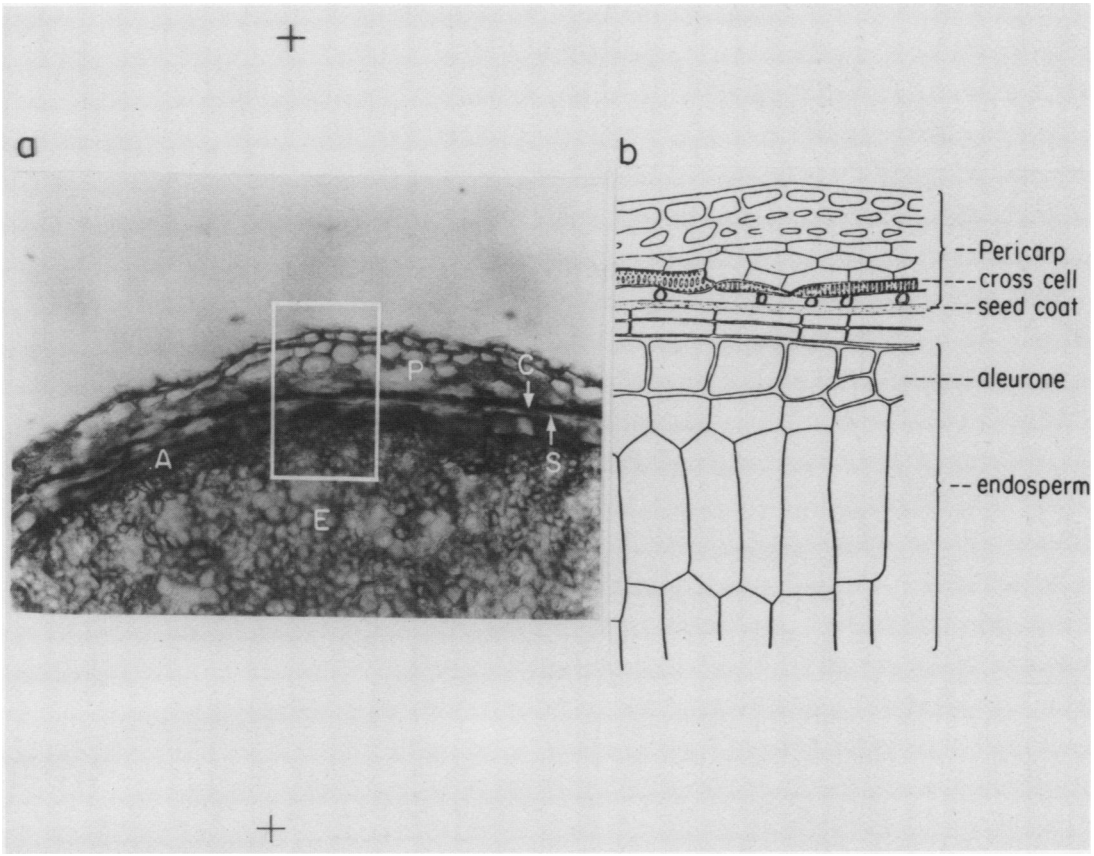

FIG. 2. Morphology of wheat seed cross section. (a) Light micrograph of the cryostat section was stained with toluidine blue (0.05%). (b) Diagram of the cross section enlarging the area within the square marked in (a). A, Aleurone layer; C, cross cell; E, endosperm; P, pericarp; and S, seed coat.

been controversial (9, 18, 21). One of the authors examined this question earlier by Western blot analysis of dissected seed parts (4). Both pericarp (photosynthetic) and endosperm contained PPDK, in contrast only pericarp contained RUBISCO (EC 4.1.1.39), consistent with our expectation of RUBISCO's role in photosynthesis. However due to the difficulty in dissection of pericarp without contamination of the aleurone layer, the previous reports were not quantitatively satisfactory.

Here we provide the first clear evidence of localization of PPDK polypeptide and mRNA in wheat seed. The validity of our method is checked by inclusion of rbcS as a control. Since the majority, if not all, of PPDK polypeptide and mRNAs are found in the aleurone layer, our results suggest a new role of PPDK in seed metabolism different from its well established role in the C4 leaf chloroplast.

MATERIALS AND METHODS

Plants. Wheat (*Triticum aestivum* L. var ERA) and maize (*Zea mays* L. cv Golden bantam) were grown in a growth chamber with a photoperiod of 16 h and at 24°C. Wheat leaves were harvested about 5 d after planting and seeds were collected 25 d after flowering. Maize leaves were harvested from 5–6 week old plants and seeds were collected from a maturing ear of corn. All plant tissues were frozen in liquid N_2 and stored at −80°C until used.

Northern Blot. Total RNA and poly(A) RNA were extracted according to the method previously described (3). RNAs from leaves and seeds were fractionated on 1.5% agarose gel containing formaldehyde (11), blotted to nitrocellulose paper, and hybridized to nick-translated PPDK cDNA in a solution containing 5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 1 × Denhardt's solution, 0.05% $Na_4P_2O_7$, 100 μg/ml denatured salmon sperm DNA, 10% (w/v) dextran sulfate at 42°C for 24 h. The blot was washed three times at room temperature in 2 × SSC, 0.1% SDS, and 0.05% $Na_4P_2O_7$ for 20 min each, then washed at 50°C in 0.5 × SSC, 0.1% SDS for 30 min. After drying in air the blot was exposed to Kodak X-AR film with an intensifying screen at −80°C for autoradiography.

Immunohistochemistry. Antibody to PPDK was prepared as described previously (2). Antibody to the small subunit of RUBISCO (rbcS was a gift of Dr. Y. Sasaki of Kyoto University). Antibodies were characterized by protein blot for their monospecificity and their titers were determined by serial dilutions.

Frozen wheat seeds were taken out from the −80°C freezer and equilibrated to the temperature (−20°C) of the cryostat (IEC; model Minotome) prior to sectioning. Wheat seeds were quickly frozen in O.C.T. compound (Tissue Tec) at −23°C. Cross sections of wheat seeds, 14 μm in thickness, were attached to glass slides coated with poly-L-lysine to prevent loss of tissues during treatment. The sections were dried on a slide warmer for 1 h, then treated with 70% ethanol for 30 min (12). The sections were rinsed in PBS (pH 7.0) three times before incubation with antibodies diluted to 1:1000 with PBS (pH 7.0) at room temperature for 5 h to overnight. After incubation, the sections were washed in PBS three times for 5 min each and then incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Miles Scientific) diluted 1:50 with PBS for 1 h at room temperature. The sections were washed thoroughly in PBS, before observing under a fluorescence microscope.

Photographs were taken within the same day using a Nikon OPTIPHOT microscope and camera equipped with fluorescence filters. Control sections were treated with nonimmunized rabbit serum, but otherwise they were treated in the same manner.

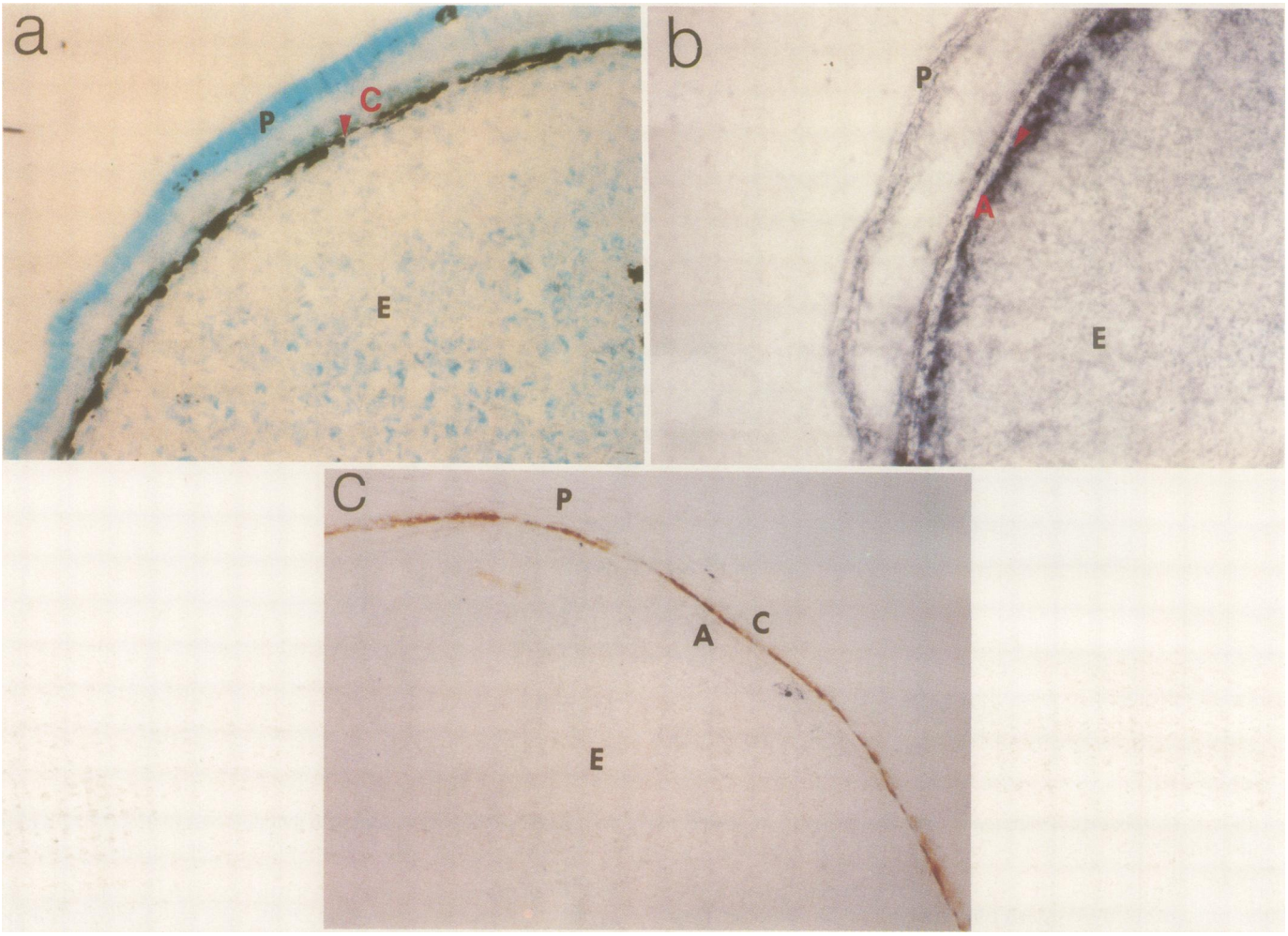

FIG. 3. *In situ* hybridization of PPDK mRNA and rbcS mRNA. The cyrostat sections were post-fixed and hybridized with ^{35}S labeled wheat rbcS cDNA (a), PPDK cDNA (b) or pBR322 DNA (c). The silver grains are found most densely around the pericarp containing Chl (cross cell) when probed with the wheat rbcS cDNA (a). There was intense staining in the aleurone layer and light staining in the pericarp when probed with the PPCK cDNA (b). Control sections (c) showed a brown line due to the discolored cross cells and not due to silver grains. A, Aleurone layer; C, cross cell; E, endosperm; and P, pericarp.

***In Situ* mRNA Hybridization.** Preparation of tissue sections is the same as in the case of immunohistochemistry up to the drying state on a slide warmer. The sections were post-fixed in 4% (w/v) paraformaldehyde in PBS (pH 7.0) for 20 min, washed in PBS, dehydrated in graded ethanol series; 70%, 95% and 100% respectively, for 3 min each, then air dried completely. The sections were pretreated with 0.2 N HCl for 20 min, rinsed in water and in 2 × SSC. Next, tissues were digested with 1 μg/ml proteinase K in 0.1 M Tris (pH 7.4), 50 mM EDTA at 37°C for 30 min, rinsed in water and in 2 × SSC twice each. The sections were dehydrated in graded ethanol series as already described above, then allowed to air dry. The tissues were prehybridized at room temperature for 1 or 2 h. The prehybridization solution contained 5 × SSC, 50% formamide, 1 × Denhardt's solution, 10% (w/v) dextran sulfate, 50 mM DTT, 100 μg/ml yeast tRNA, 100 μg/ml denatured salmon sperm DNA. For use as probes, PPDK cDNA (11), wheat rbcS cDNA (6) and pBR322 DNA were labeled with ^{35}S- or ^{32}P-dNTP (Amersham) by nick-translation to specific activities above 1×10^8 cpm/μg. About 5×10^5 cpm per slide were used. The hybridization was done at 37°C for 36 h. The sections were washed in 2 × SSC, 5 mM DTT, 0.1 % SDS at room temperature once for 10 min and twice for 30 min. This was followed by washing in 1 × SSC, 5 mM DTT, and 0.1% SDS for 30 min at 40°C, then in the same buffer of a total volume of 4 L of solution for 2 h at room temperature. The sections were dehydrated in graded ethanol solutions (70%, 95%, 100%) containing 0.3 M ammonium acetate (8) then air dried.

Slides were coated with Kodak NTB2 emulsion at 45°C and allowed to dry slowly. After drying, they were placed in dark boxes with dessicant for exposure at 4°C for various time periods. The slides were developed using D19 developer at 15°C for 2.5 min, rinsed in water briefly and placed in fixer for 10 min. After fixing, the slides were then rinsed in water for 30 min with frequent changes of water. Some slides were stained with toluidine blue and mounted with coverslips.

RESULTS

Immunofluorescent Labeling of PPDK and rbcS within Wheat Seed. When wheat seeds were probed with antibody to the rbcS polypeptide the area containing chlorophyll in the pericarp showed intense fluorescence (Fig. 1a). When probed with antibody to PPDK, the aleurone layer showed strong fluorescence with some weak fluorescence in the pericarp (Fig. 1b). No fluorescence was detected in the control sample with nonimmunized serum (data not shown).

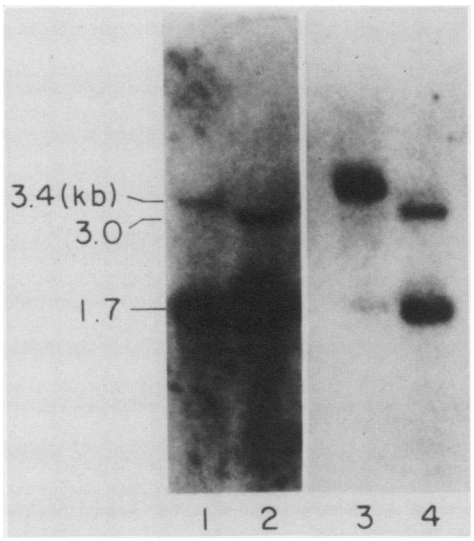

FIG. 4. Northern blot hybridization analysis of PPDK mRNA organ-specificity in leaves and seeds of maize and wheat. Total RNA samples from wheat leaves (20 μg lane 1) wheat seeds (20 μg, lane 2) maize leaves (5 μg, lane 3) and maize seeds (10 μg, lane 4) were subjected to electrophoresis in a 1.5% agarose:formaldehyde gel. The blot was hybridized with ^{32}P-labeled PPDK cDNA. The *arrows* indicate sizes of mRNAs. Autoradiography was 3 d for maize and 7 d for wheat.

***In Situ* Hybridization of PPDK mRNA and rbcS mRNA.** Previous studies have demonstrated that the wheat seed PPDK mRNA activity changes with development and reaches a maximum at 25 d after flowering (4). Seeds at this developmental stage were therefore used in our experiments.

The silver grains which indicate the hybridization are most dense in the chlorophyllous pericarp (Fig. 2) for rbcS mRNA (Fig. 3a) and in the aleurone layer for PPDK mRNA (Fig. 3b). The signal is specific, because no signal was obtained when nick-translated pBR322 was used as a probe (Fig. 3c). This is the first report to localize PPDK and rbcS mRNAs by *in situ* hybridization in wheat seed.

Organ-Specific Expression of PPDK mRNA in Wheat. The maize PPDK cDNA probe hybridizes with mRNAs of the size 3.4 kb from both maize and wheat leaves (Fig. 4). In contrast, it hybridizes to mRNA of 3.0 kb from maize and wheat seeds, similar to the value reported for maize root (13). The presence of organ-specific mRNA is consistent with results obtained by *in vitro* translation of mRNAs followed by immunoprecipitation with PPDK antibody (3). The clear hybridization suggests that PPDK structure is highly conserved between wheat (C3) and maize (C4). In wheat leaf and seed there is an additional 1.7 kb band (Fig. 4, lanes 1 and 2). This mRNA is abundant in wheat leaf and seed grown in the dark or light (data not shown), but in maize leaf and seed this mRNA band of similar size is only visible after prolonged exposure.

DISCUSSION

Localization of PPDK and rbcS in Wheat Seed. The study of gene expression of tissue-specific and developmentally regulated enzymes in plants has been done with plant homogenates using biochemical assays, protein, DNA or RNA blot analyses in the past. The precise localization of PPDK in dissected seed at 25 d after flowering is very difficult, since peeling the pericarp without contamination of the aleurone layer becomes harder as the seed reaches maturity (Fig. 2). We have studied gene expression using both *in situ* immunofluorescent labeling and *in situ* mRNA hybridization in plant tissue sections. Cellular specificity was verified by conducting *in situ* hybridization in conjunction with immunohistochemistry with rbcS, which was expected to be confined to chlorophyllous pericarp tissue (4, 9, 21). In the present report, PPDK polypeptide and mRNA are found predominantly in the aleurone layer with some in the pericarp.

Organ-Specific PPDK mRNA. In wheat, there are organ-specific mRNAs as in maize. The largest mRNA is about 3.4 kb found in leaf and 3.0 kb found in seed. The difference in size of 400 bases corresponds to the size of RNA required to encode 16 kD transit polypeptide which is needed for chloroplastic PPDK polypeptide to enter into chloroplast after being synthesized in the cytoplasm (5). The third mRNA band of 1.7 kb is too small to encode PPDK which is reported to be a tetramer with subunits of approximately 94 to 97 kD (20). The 1.7 kb mRNA is not unique to wheat leaf as shown here and by Hudspeth *et al.* (13); who observed a similar size RNA in etiolated maize leaf and root RNAs. The intensity of 1.7 kb band is similar in dark grown and light grown wheat leaf, whereas the 3.4 kb mRNA level is light regulated (13). Since the same mRNA preparation gave single bands on Northern blot when hybridized with other DNA probes (14), the 1.7 kb band is not due to a general degradation of mRNA. Therefore the 1.7 kb mRNA may be transcribed from a different member of the gene family. Alternatively, it could be a product of differential transcription from the same gene using different starting sites or a result of post-transcriptional processing of the 3.4 kb mRNA. We are investigating these points further.

The metabolic role of PPDK in wheat seed may be in providing PEP for PEPC. At the late stage of wheat seed development rbcS declines in quantity (4, 18) and there is almost 100 times more PEPC activity than rbcS activity (9). Since there are only a few stomata in wheat pericarp, the entry of atmospheric CO_2 into the chlorophyllous tissue is restricted by the cuticle (Fig. 2) (16). PEPC may therefore refix respired CO_2 to prevent carbon loss. There are high levels of aspartate amino transferase, glutamate-oxaloacetate transaminase (21), ADP-malate dehydrogenase glutamine synthetase (9) in pericarp tissue, but NADP-malic enzyme activity is low (21).

C4 acids formed therefore may not be decarboxylated to pyruvate and CO_2 which is refixed by rbcS as in the case of the C4 photosynthetic pathway. Instead, they may be used to contribute to the synthesis of seed storage protein as previously suggested (4). ATP needed for the PPDK reaction can be supplied by adjoining mitochondria in the aleurone layer (19), independent of the photosynthetic ATP synthesis. This is also consistent with the report that extractable wheat seed PPDK activity was unchanged when the seeds developed without exposure to light (18). Our results on PPDK localization in nonphotosynthetic tissue indicate a distinct role of PPDK different from that in C4 leaf chloroplast.

The method we outlined here using frozen cut serial sections allows the detection of both polypeptides and mRNA in a single sample.

Our work indicates that cell-specific accumulation of PPDK and rbcS polypeptide and mRNA is likely due to their cell-specific mRNA accumulation. Use of *in situ* hybridization is still limited in plant biology (1, 17), although it has been used widely by animal researchers (8, 10). We believe that this method is a powerful tool for studying plant gene expression at the cell level.

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LITERATURE CITED

1. ANDERSON MA, EC CORNISH, SL MAU, EG WILLIAMS, R HOGGART, A ATKINSON, I BONIG, B GREGO, R SIMPSON, J ROCHE, JD HALEY, JD PENSCHOW, HD NIALL, GW TREGEAR, JP COGHLAN, RJ CRAWFORD, AE CLARKE 1986 Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. Nature 321: 38–44
2. AOYAGI K, JA BASSHAM 1984 Pyruvate orthophosphate dikinase in wheat leaves. Plant Physiol 73: 853–854
3. AOYAGI K, JA BASSHAM 1984 Pyruvate orthophosphate dikinase mRNA organ specificity in wheat and maize. Plant Physiol 76: 278–280
4. AOYAGI K, JA BASSHAM, FC GREENE 1984 Pyruvate orthophosphate dikinase gene expression in developing wheat seeds. Plant Physiol 75: 393–396
5. AOYAGI K, JA BASSHAM 1985 Synthesis and uptake of cytoplasmically synthesized pyruvate, Pi dikinase polypeptide by chloroplasts. Plant Physiol 164: 207–214
6. BROGLIE R, G CORUZZI, G LAMPPA, B KEITH, N-H CHUA 1983 Structural analysis of nuclear genes coding for the precursor to the small subunit of wheat ribulose-1,5-bisphosphate carboxylase. BioTechnology 1: 55–61
7. BURNELL JN, MD HATCH 1985 Regulation of C4 photosynthesis: purification and properties of the protein catalyzing ADP-mediated inactivation and Pi-mediated activation of pyruvate, Pi dikinase. Arch Biochem Biophys 237: 490–503.
8. COX KH, DV DELEON, LM ANGERER, RC ANGERER 1984 Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. Dev Biol 101: 485–502
9. DUFFUS CM, R ROSIE 1973 Some enzyme activities associated with the chlorophyll containing layers of immature barley pericarp. Planta 114: 219–226
10. HAFEN E, M LEVINE, RL GARBER, WJ GEHRING 1983 An improved *in situ* hybridization method for the detection of cellular RNAs in Drosophila tissue sections and its application for localizing transcripts of the homeotic *Antennapedia* gene complex. EMBO J 2: 617–623
11. HAGUE DR, M UHLER, PD COLLINS 1983 Cloning of cDNA for pyruvate, Pi dikinase from maize leaves. Nucleic Acid Res 11: 4853–4856
12. HATTERSLEY PW, L WATSON, CB OSMOND 1977 *In situ* immunofluorescent labelling of ribulose-1,5-bisphosphate carboxylase in leaves of C3 and C4 plants. Aust J Plant Physiol 4: 523–539
13. HUDSPETH R, CA GLACKIN, J BONNER, JW GRULA 1986 Genomic and cDNA clones for maize phosphoenolpyruvate carboxylase and pyruvate, orthophosphate dikinase: expression of different gene-family members in leaves and roots. Proc Natl Acad Sci USA 83: 2884–2888
14. KEITH B, N-H CHUA 1986 Monocot and dicot pre-mRNAs are processed with different efficiencies in transgenic tobacco. EMBO J 5: 2419–2425
15. KISAKI T, S HIRABAYASHI, N YANO 1973 Effect of age of tobacco leaves on photosynthesis and photorespiration. Plant Cell Physiol 14: 505–514
16. KRIEDEMANN P 1966 The photosynthetic activity of the wheat ear. Ann Bot 30: 349–362
17. MARTINEAU B, WC TAYLOR 1986 Cell-specific photosynthetic gene expression in maize determined using cell separation techniques and hybridization *in situ*. Plant Physiol 82: 613–618
18. MEYER AO, GJ KELLY, E LATZKO 1982 Pyruvate orthophosphate dikinase from immature grains of cereal grasses. Plant Physiol 69: 7–10
19. OPARKA KJ, PJ GATES, D BOULTER 1981 Regularly aligned mitochondria in aleurone and subaleurone layers of developing rice caryopses. Plant Cell Environ 4: 355–357
20. SUGIYAMA T 1973 Purification, molecular, and catalytic properties of pyruvate phosphate dikinase from maize leaf. Biochem 12: 2862–2868
21. WIRTH E, GJ KELLY, G FISCHBECK, E LATZKO 1977 Enzyme activities and products of CO_2 fixation in various photosynthetic organs of wheat and oat. Z Pflanzenphysiol 82: 78–87