Genomic and cDNA clones for maize phosphoenolpyruvate carboxylase and pyruvate,orthophosphate dikinase: Expression of different gene-family members in leaves and roots

(photosynthesis/enzymes/light-regulation/tissue specificity)

RICHARD L. HUDSPETH, CARLOTTA A. GLACKIN, JAMES BONNER, AND JOHN W. GRULA*

Phytogen, 101 Waverly Drive, Pasadena, CA 91105

Contributed by James Bonner, December 18, 1985

ABSTRACT We have isolated cDNA clones for the maize leaf enzymes phosphoenolpyruvate (P-ePrv) carboxylase [orthophosphate:oxaloacetate carboxy-lyase (phosphorylating) EC 4.1.1.31] and pyruvate,orthophosphate (Prv,Pi) dikinase (ATP:pyruvate,orthophosphate phosphotransferase, EC 2.7.9.1) by exploiting the light-inducibility and large size of the mRNAs (3.5 kilobases) that encode the two enzymes. The clones were identified by hybrid-selection and immunoprecipitation assays. From a maize genomic library, two different types of genomic clones were screened with both the P-ePrv carboxylase and the Prv,Pi dikinase cDNA clones. Information from these genomic clones and genome blots indicates that the P-ePrv carboxylase gene family has at least three members and the Prv,Pi dikinase gene family at least two. Transcripts for both enzymes were detected in green leaves, etiolated leaves, and roots. The results show that the P-ePrv carboxylase mRNAs in green leaves and roots are encoded by different genes. Whereas the P-ePrv carboxylase mRNAs in all three tissues appear to be the same size, the Prv,Pi dikinase mRNA in green leaves is about 0.5 kilobases longer than the Prv,Pi dikinase mRNAs in etiolated leaves and roots. It is possible that all these Pv,Pi dikinase transcripts are encoded by one gene, and the size differences may correspond to the presence or absence of a sequence encoding a chloroplast transit peptide.

The enzymes phosphoenolpyruvate (P-ePrv) carboxylase [orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31] and pyruvate,orthophosphate (Prv,Pi) dikinase (ATP:pyruvate,orthophosphate phosphotransferase, EC 2.7.9.1) play important roles in C4 and crassulacean acid metabolism photosynthesis. P-ePrv carboxylase is responsible for the fixation of atmospheric CO2 while Prv,Pi dikinase produces the substrate phosphoenolpyruvate for P-ePrv carboxylase (1). In green leaves of the C4 plant maize (Zea mays), P-ePrv carboxylase is located in the cytoplasm of mesophyll cells (2). Prv,Pi dikinase is found primarily in the chloroplasts of mesophyll cells (3–5), although some is also detectable in bundle-sheath cells (6). The former enzyme has a subunit molecular mass of 100–103 kDa and has been estimated to comprise 8–15% of the total leaf soluble protein (7–9). The latter enzyme has a subunit molecular mass of 94–97 kDa and makes up 2–10% of the total leaf soluble protein (10–12). Maximal accumulation of both of these enzymes and their mRNAs is light-dependent (1, 8, 11, 13–15). In addition, P-ePrv carboxylase has been found in other maize tissues, including etiolated leaves and roots (8, 14–16), and Prv,Pi dikinase has been detected in maize seeds (17) and etiolated leaves (13). However, these forms of the enzymes have received much less study.

Here we describe the isolation and partial characterization of P-ePrv carboxylase and Prv,Pi dikinase cDNA and genomic clones from maize. Our results indicate that each enzyme is encoded by a small number of genes that exhibit differential expression in leaves and roots.

MATERIALS AND METHODS

Growth and Harvesting of Plant Material. Maize (Zea mays) plants were grown from both inbred (B73) and hybrid (Golden Jubilee) varieties of seed. DNA and the poly(A)+ RNA used to construct the leaf cDNA library was extracted from B73 plants. All other RNAs were obtained from Golden Jubilee. Green leaves and roots were harvested from 10- to 14-day-old and 60-day-old plants, respectively, that had been grown in a room illuminated with high-intensity mercury-vapor lamps (16 hr light/8 hr dark). Etiolated leaves, fully emerged from the coleoptile, were obtained from 14-day-old plants that had been germinated, grown, and harvested in complete darkness.

Isolation and Labeling of Nucleic Acids. Total maize DNA was extracted from etiolated leaves (18), phage DNAs were isolated according to Maniatis et al. (19), and plasmid DNAs were prepared by the protocol of Norgard (20). Total RNA was extracted from maize tissues by the guanidinium thiocyanate procedure (21), and the poly(A)+ fraction was isolated by one pass over oligo(dT)-cellulose (19). Oligo(dT)-primed cDNA probes were prepared according to the procedure of Maniatis et al. (19), and other DNA probes were labeled by nick-translation (22).

Construction and Screening of cDNA and Genomic Libraries. Double-stranded DNA copies of total poly(A)+ RNA from green maize leaves were synthesized according to the protocol of Efstratiadis et al. (23) and inserted into the Pst I site of pBR322 (24). The library was screened with various cDNA probes by the colony-hybridization method (25). To construct a genomic library, total maize DNA was partially digested with Mbo I and fragments of 15–20 kilobase pairs (kb) were ligated to BamHI-digested vector arms from EMBL3 (26). The recombinant phage were plate-amplified (27) and then screened by plaque hybridization (28).

Hybrid-Selection and Immunoprecipitation Procedures. Plasmid DNAs used for hybrid selection were bound to nitrocellulose disks (0.5 cm) as described by Kafatos et al. (29). Hybridization and washing of the disks and elution of bound RNA were performed according to Maniatis et al. (19), except the hybridization temperature was adjusted to 45°C. For each hybrid-selection, 25 µg of total poly(A)+ RNA from green leaves was hybridized at a concentration of 500 µg/ml.

Abbreviations: P-ePrv carboxylase, phosphoenolpyruvate carboxylase; Prv,Pi dikinase, pyruvate,orthophosphate dikinase; kb, kilobase(s); bp, base pair(s).

*To whom reprint requests should be addressed.
to two disks, each containing 20 μg of the same plasmid DNA. The hybridized RNA that was eluted from the filters was translated in rabbit reticulocyte lysate (Promega Biotech, Madison, WI) in the presence of [35S]methionine and [3H]-leucine.

Immunoprecipitation of in vitro translation products with Staphylococcus aureus Cowan 1 strain cells (IgGorb; The Enzyme Center, Boston) was performed as described by Cullen and Schwartz (30). Antibodies against maize P-ePrv carboxylase (obtained from Sigma) were prepared by Antibodies Inc. (Davis, CA). Maize Prv,Pi dikinase antiserum was a gift of T. Sugiyama (Nagoya University, Chikusa, Nagoya, Japan).

Proteins were analyzed by NaDodSO4/polyacrylamide gel electrophoresis in 5–15% gradient slab gels run at 3 V/cm for 16 hr (31). The gels were treated with ENHANCE (New England Nuclear), dried, and exposed for 16–72 hr to x-ray film at −70°C with an intensifying screen.

Gel Blot Hybridization Procedures. DNA fragments were transferred from agarose gels to nitrocellulose or Zeta-Probe membranes (Bio-Rad) and hybridized with DNA probes as described by Kleissig and Berry (32). RNAs were transfected to nitrocellulose from agarose/formaldehyde gels as described by Maniatis et al. (19). To ensure that equal amounts of different poly(A)+ RNAs were loaded in gel lanes, the concentrations were quantified with a poly([3H]U) hybridization assay (33). Unless otherwise indicated, DNA and RNA blots were hybridized at 42°C in a solution containing 50% (vol/vol) formamide, and final washes were carried out at 52°C in 30 mM NaCl/3 mM sodium citrate/0.1% (wt/vol) N-lauroyl sarcosine (32).

RESULTS

Isolation and Partial Characterization of cDNA and Genomic Clones. Three different criteria were used to select potential P-ePrv carboxylase and Prv,Pi dikinase clones from a 1000-member maize leaf cDNA library: (i) greater reactivity to a cDNA probe made from green leaf poly(A)+ RNA than to a cDNA probe made from etiolated leaf poly(A)+ RNA, (ii) a positive reaction to a cDNA probe made from green leaf poly(A)+ RNA enriched for P-ePrv carboxylase and Prv,Pi dikinase mRNA by size fractionation, and (iii) a blot-hybridization reaction with a green leaf mRNA of adequate size to encode the P-ePrv carboxylase and Prv,Pi dikinase polypeptides [=3 kilobases (kb)].

Two cDNA clones, designated pH1 and pH2, were isolated through this selection process. Preliminary hybrid-selection assays indicated that pH1 was a P-ePrv carboxylase clone and pH2 was possibly a Prv,Pi dikinase clone. On the basis of these results, we screened a maize genomic library with both clones. Analysis of the isolated genomic clones showed that pH1 hybridizes to a set of clones that is different from the set to which pH2 hybridizes. In addition, we found that two different types of genomic clones had been screened with each cDNA clone. These genomic clones can be distinguished by their different restriction endonuclease maps (Fig. 1) and their different intensities of hybridization with each cDNA clone. Fig. 2 shows that pH1 hybridizes more intensely with H1X14 than it does with H1X21, whereas pH2 hybridizes more intensely with H2X13 than it does with H2X23. These differences in hybridization are probably due to differences in base sequence homology, and, if so, indicate that H1X14 and H2X13 have greater homology with the respective cDNA clones than do H1X21 and H2X23. In addition, Fig. 2 shows that four HindIII fragments in the maize genome hybridize to pH1, of which two are accounted for by HindIII fragments in H1X14 and H1X21. The number of genomic HindIII fragments observed hybridizing to pH2 is two, and these correspond to HindIII fragments in H2X13 and H2X23. The genomic clone reconstructions in Fig. 2 indicate that the corresponding genomic fragments are present in 1–2 copies per haploid genome.

Clone Identification by Hybrid-Selection and Immunoprecipitation Assays. In order to increase the length of sequence that could hybridize to mRNA, genomic subclones pH1X14-13 and pH2X13-4 were subsequently used for additional hybrid-selection experiments. Genomic subclone pH1X14-13 contains a 13-kbp insert that hybridizes to the pH1 cDNA clone, and genomic subclone pH2X13-4 contains a 4-kbp insert that hybridizes to the pH2 cDNA clone (Fig. 1).

![Fig. 1](image-url)  Representative examples of the two types of genomic clones that were screened with both the pH1 (A; P-ePrv carboxylase) and pH2 (B; Prv,Pi dikinase) cDNA clones. The cDNA-clone inserts are shown above the genomic-clone restriction endonuclease fragments to which each hybridized. The genomic subclones used for hybrid selection and as RNA blot probes (pH1X14-13 and pH2X13-4) are indicated directly below the maps of H1X14 and H2X13. Restrictions sites: E, EcoRI; H, HindIII; S, SalI; B, BamHI; K, KpnI; X, XhoI. Sites enclosed in parentheses map to one of the two positions indicated.
The p-prv carboxylase antibody, from a sample equivalent to that in lane 7, with P-ePrv carboxylase antibody (some Prv,Pi dikinase was also immunoprecipitated, probably because the P-ePrv carboxylase used to elicit the antibody was contaminated with Prv,Pi dikinase). Lane 9: immunoprecipitate from a sample equivalent to that in lane 7, with Prv,Pi dikinase antibody. Molecular mass markers are indicated at left in kDa. The arrowhead shows the position of a 103-kDa P-ePrv carboxylase marker.

Fig. 3 shows the data used to identify pH1 as a P-ePrv carboxylase clone and pH2 as a Prv,Pi dikinase clone. The mRNA selected by pH1A14-13 encodes a polypeptide that comigrates with the 103-kDa P-ePrv carboxylase marker (lane 2). This is the size of both the in vitro- and in vivo-synthesized protein subunit (8, 14). The 103-kDa polypeptide in lane 2 is also immunoprecipitated by P-ePrv carboxylase antibody (lane 5).

The mRNA selected by pH2A13-4 codes for a 110-kDa polypeptide (Fig. 3, lane 3), which is the size of the Prv,Pi dikinase subunit precursor synthesized in vitro (11, 34). The size difference between the in vitro translation product (110 kDa) and the 94- to 97-kDa subunit found in vivo is probably accounted for by a transit peptide which is removed during the transport of Prv,Pi dikinase from the cytoplasm into the chloroplasts of mesophyll cells (5, 11, 34). Immunoprecipitation of the 110-kDa polypeptide by Prv,Pi dikinase antibody is shown in lane 6. Characterization of the P-ePrv carboxylase and Prv,Pi dikinase antibodies is shown in lanes 7–9. From these results, we conclude that pH1 is a P-ePrv carboxylase cDNA clone and pH2 a Prv,Pi dikinase cDNA clone.

P-ePrv Carboxylase and Prv,Pi Dikinase mRNA Expression in Maize Leaves and Roots. When the P-ePrv carboxylase cDNA clone (pH1) is used to probe a blot of electrophoretically fractionated poly(A)+ RNA from green leaves, an intense reaction with an mRNA of about 3.5 kb is observed. There is no detectable reaction of pH1 with any transcripts in poly(A)+ RNA from etiolated leaves or roots (Fig. 4A). These results corroborate a previous report (15) that showed that maximal accumulation of one or more forms of 3.5-kb P-ePrv carboxylase mRNA is induced by light in maize leaves.

When the genomic subclone pH1A14-13 is used to probe the same RNAs, a similar pattern of hybridization to that observed with the pH1 probe is obtained, except very weak reactions can also be detected to 3.5-kb transcripts in the etiolated leaf and root RNAs (Fig. 4B). The greater sensitivity obtained with the pH1A14-13 probe is due at least in part to the fact that it contains a substantial amount of a P-ePrv carboxylase gene coding region (=3 kb; unpublished data), whereas the hybridizable sequence in pH1 is much smaller (400 bp).

In contrast to pH1 and pH1A14-13, the genomic clone H1A21 exhibits a very different pattern of hybridization to the leaf and root RNAs (Fig. 4C). H1A21 reacts most strongly with the root mRNA, while exhibiting weaker and approximately equal reactions with the green and etiolated leaf mRNAs. Clearly, the P-ePrv carboxylase mRNAs in green leaves and roots are encoded by different genes that differ substantially in base sequence. Further, these results provide evidence that pH1A14-13 contains genomic sequences that encode the light-inducible mRNA in green leaves, and that H1A21 contains sequences encoding root mRNA. The data in Fig. 4B and C also suggest that the mRNA in etiolated leaves may be encoded by a third type of P-ePrv carboxylase gene.

Like the P-ePrv carboxylase cDNA clone (pH1), the Prv,Pi dikinase cDNA clone (pH2) also reacts intensely with a
3.5-kb mRNA in green leaves (Fig. 5A). pH2 also reacts weakly with larger transcripts in green leaves, which may be precursors of the 3.5-kb species. Whereas pH1 does not react detectably with etiolated leaf and root RNA, pH2 does hybridize weakly to transcripts in both of these tissues (Fig. 5A). The intensity of these reactions is not enhanced by reducing the hybridization and wash temperatures by 12°C (data not shown). Therefore, these weak signals are probably due to the low abundance of the Prv,P dikinase transcripts in these tissues and not due to sequence divergence. These results are consistent with an earlier study (11) and indicate that one or more forms of Prv,P dikinase mRNA in green leaves are induced by light to become much more abundant than the Prv,P dikinase transcripts in etiolated leaves and roots.

When the genomic clones pH2A13-4 and H2A23 are used to probe the two types of leaf poly(A)+ RNA and root poly(A)+ RNA, a pattern of hybridization similar to that of the pH2 probe is observed (Figs. 5 B and C). Compared to pH2A13-4, H2A23 reacts less intensely with the Prv,P dikinase transcripts in all three tissues (on a longer exposure, reaction with the etiolated leaf and root Prv,P dikinase transcripts by H2A23 becomes clearly visible). Because the specific activities of the two probes were very similar, this difference is probably due to H2A23 having less homology with these mRNAs than does pH2A13-4. Thus, it is possible that the Prv,P dikinase transcripts detected in all three tissues are encoded by the gene represented by pH2A13-4. The function of the Prv,P dikinase gene sequence in H2A23 is unclear; two possibilities are that it (i) codes for an mRNA that is expressed in a tissue or cell type not analyzed here or (ii) contains a pseudogene that has diverged from the sequence of the leaf and root transcripts.

The RNA blot results shown in Fig. 5 also suggest that Prv,P dikinase transcripts exist in etiolated leaves and roots that may be smaller than the 3.5-kb mRNA in green leaves. (Actually two transcripts can be detected in lanes E of Fig. 5 A and B, one that is about 3.5 kb long and one that is smaller.) As a consequence, the blot hybridizations in Fig. 6 were carried out to verify whether or not size differences exist among the P-ePrv carboxylase and Prv,P dikinase messages in leaves and roots. The P-ePrv carboxylase genomic subclone (pH1A14-13) and the Prv,P dikinase cDNA clone (pH2) were used to probe blots in which a reduced amount of green leaf poly(A)+ RNA (25 ng) was mixed with 1 μg of root poly(A)+ RNA, and the two were electrophoresed together in the same lane. In adjacent lanes, 1 μg of root and etiolated leaf poly(A)+ RNA and 25 ng of green leaf poly(A)+ were electrophoresed separately (Fig. 6). No size differences were detected among the different P-ePrv carboxylase transcripts (Fig. 6A), but the results clearly show that etiolated leaves and roots contain Prv,P dikinase transcripts ~0.5 kb shorter than green leaf mRNA (3.0 kb vs. 3.5 kb; Fig. 6B). In the lanes containing root poly(A)+ RNA, an unidentified transcript too small (~1.7 kb) to encode any known form of Prv,P dikinase is also visible.

The RNA blot in Fig. 6B also shows that the Prv,P dikinase mRNA in green leaves is at least 40-fold more abundant per μg of poly(A)+ RNA than the root and etiolated leaf transcripts. This is concluded because the green leaf mRNA produced the strongest signal, yet 1/40th as much green leaf poly(A)+ RNA was probed compared to the other two RNAs (25 ng vs. 1 μg).

**DISCUSSION**

We have demonstrated that the maize enzymes P-ePrv carboxylase and Prv,P dikinase are encoded by small multigene families with members that exhibit differential expression in green leaves, etiolated leaves, and roots. Until all the sequences that hybridize with P-ePrv carboxylase and Prv,P dikinase coding-region probes are cloned, it will not be possible to specify the exact number of genes in each family. Simply estimating from the number of genomic HindIII fragments observed to hybridize to each cDNA clone (Fig. 2), we can place a lower limit on the number of P-ePrv carboxylase genes at three or four and the number of Prv,P dikinase genes at two. In the case of P-ePrv carboxylase, it is clear that different genes encode the mRNAs in green leaves and roots and that these genes differ substantially in base sequence (Fig. 4). This substantial sequence divergence among P-ePrv carboxylase gene-family members may explain a previous report of protein accumulation in the absence of detectable mRNA (15); the detectability of different P-ePrv carboxylase mRNAs is clearly probe-dependent. The data in Fig. 4 also suggest that the mRNA in etiolated leaves may be transcribed from a third member of the P-ePrv carboxylase gene family.

In the case of Prv,P dikinase, it is possible that one gene or two very similar genes (represented by genomic clone H2A13) encode the mRNAs detected in green leaves, etiolated leaves, and roots. The genomic-duplicate and genome-blot information (Figs. 1 and 2) provide evidence for only one other type of Prv,P dikinase gene (represented by H2A23). This gene appears to have diverged substantially from the leaf and root transcripts, and it may encode an mRNA expressed in a tissue not analyzed here or it may be a pseudogene. If one gene does encode all the Prv,P dikinase transcripts in leaves...
and roots, different transcription start or stop sites and/or differential RNA processing would be required to account for the different sizes of the green leaf mRNA (3.5 kb) versus etiolated leaf and root mRNAs (3.0 kb).

The Prv,P1 dikinase transcript-size differences are interesting in the context of known differences in the size of the Prv,P1 dikinase polypeptide precursors in photosynthetic versus nonphotosynthetic tissues (34). Chloroplast uptake studies and comparisons between in vitro translation products and mature subunits found in vivo provide evidence that the precursors in maize and wheat green leaves have a transit peptide, about 16 kDa in size, that the precursors in maize and wheat seeds lack (5, 11, 34). The 0.5-kb size difference we observe between Prv,P1 dikinase transcripts in photosynthetic and nonphotosynthetic tissues corresponds closely to the amount of RNA required to encode 16 kDa of polypeptide. These mRNA size differences may therefore be accounted for by the presence or absence of the sequence that encodes the transit peptide.

Light is required for maximal accumulation of the P-ePrv carboxylase and Prv,P1 dikinase isozymes involved in C4 photosynthesis (1, 8, 13), and as shown here and in other studies (11, 14, 15), maximal accumulation of P-ePrv carboxylase and Prv,P1 dikinase leaf mRNA is also light-dependent. Because the genomic subclones pH1A14-13 and pH2A13-4 hybridize very strongly to the light-inducible leaf messages, it is possible that the genomic clones from which they were derived contain the genes that encode the C4-photosynthetic forms of these enzymes. Additional studies will be needed to confirm the identification of these clones and to determine the structure and function of the remaining members of the P-ePrv carboxylase and Prv,P1 dikinase gene families.

Note Added in Proof. We have recently derived amino acid sequence information from DNA sequence data obtained from the P-ePrv carboxylase clone H1A14. This information indicates that there are several regions 12–26 amino acids long in which the enzyme encoded in this clone is 70–90% homologous to Escherichia coli P-ePrv carboxylase (36).

We thank Dr. Tatsuo Sugiyama for antiserum against maize leaf Prv,P1 dikinase and Dr. Hans Lehrach for EMBL phage vectors and host strains. Drs. Richard Yenofsky, Constantin Flytzanis, and David Anderson provided valuable information on various procedures. We thank Drs. Robert Goldberg, Richard Yenofsky, and David Anderson for very helpful criticism of the work while in progress and of the manuscript. Mary Strother and Elizabeth Hanson assisted in the manuscript preparation. This work was supported by Eli Lilly and Company (Indianapolis, IN) and the J. G. Boswell Company (Los Angeles).


