Maize Phosphoenolpyruvate Carboxylase

CLONING AND CHARACTERIZATION OF mRNAs ENCODING ISOZYMIC FORMS*

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The isozymic forms of maize phosphoenolpyruvate carboxylase (P-enolpyruvate carboxylase) involved in photosynthetic CO₂ fixation were shown by protein gel blot analysis to consist of 100-kDa subunits. The non-autotrophic isozyme found in roots is comprised of 96-kDa subunits and is about 50–100-fold less prevalent. Further analysis of P-enolpyruvate carboxylase isoforms made use of cloned cDNA probes. Two cDNA clones were isolated from a library constructed from maize leaf poly(A) RNA. The largest clone was complementary to about 25% of P-enolpyruvate carboxylase mRNA, which is 3.4 kilobases in length. The quantity of P-enolpyruvate carboxylase mRNA in green, mature leaf tissue was estimated to be 0.20% of poly(A) RNA, whereas P-enolpyruvate carboxylase mRNA in roots was about 100-fold less prevalent. We used thermal denaturation of a P-enolpyruvate carboxylase cDNA probe hybridized to RNA gel blots to estimate the degree of sequence difference between mRNAs encoding different P-enolpyruvate carboxylase isoforms. There appear to be at least two prevalent P-enolpyruvate carboxylase mRNAs in green leaves which are significantly different in sequence, as are P-enolpyruvate carboxylase mRNAs in roots and shoots. The hybridization pattern of maize genomic DNA Southern blots indicates that P-enolpyruvate carboxylase is encoded by a small gene family.

Many plant species which inhabit desert or semi-arid tropical environments employ a unique form of CO₂ fixation called C₄ metabolism. A set of specialized enzymes and a specialized leaf morphology called "Kranz anatomy" provide C₄ plants with physiological adaptations to these environments. Fixation of atmospheric CO₂ and the first steps of photosynthetic carbon reduction are catalyzed by different isozymic forms of P-enolpyruvate carboxylase. Chromatographic and kinetic properties of P-enolpyruvate carboxylases isolated from dark-grown and light-grown maize leaves were found to be different as were those of P-enolpyruvate carboxylase from maize roots (10, 16, 17). Two chromatographically separable peaks of P-enolpyruvate carboxylase activity were found in leaves at different stages of development. One of these may have been a non-autotrophic form of P-enolpyruvate carboxylase because it had a Kₘ for CO₂ and the subsequent flow of the four-carbon organic acids to the bundle sheath cells acts as a "CO₂ pump" to increase the CO₂ to O₂ ratio in bundle sheath cells such that the reductive pentose phosphate cycle operates more efficiently (7, 8). In plants utilizing the more common C₄ pathway, atmospheric CO₂ is fixed directly by ribulose-1,5-bisphosphate carboxylase.

P-enolpyruvate carboxylase plays more than one metabolic role, its precise function depending on the organ and plant in which it is found. In C₄ plants, a non-prevalent form of P-enolpyruvate carboxylase fixes CO₂ with malate as an end product (9, 10). It has been proposed that the malate generated in this reaction may function in cell osmoregulation (11, 12). This is a minor reaction compared with the generation of malate as a photosynthetically intermediate in C₃ plants. P-enolpyruvate carboxylase also functions as an anaplerotic enzyme in both plants and bacteria (13, 14). The enzymatic properties of P-enolpyruvate carboxylase have been reviewed by O'Leary (15).

Previous studies have suggested that these different functions are catalyzed by different isozymic forms of P-enolpyruvate carboxylase. Chromatographic and kinetic properties of P-enolpyruvate carboxylases isolated from dark-grown and light-grown maize leaves were found to be different as were those of P-enolpyruvate carboxylase from maize roots (10, 16, 17). Two chromatographically separable peaks of P-enolpyruvate carboxylase activity were found in leaves at different stages of development. One of these may have been a non-autotrophic form of P-enolpyruvate carboxylase because it had a Kₘ for phosphoenolpyruvate similar to that of the enzyme isolated from maize roots. Recently, Harpster (18) extended the chromatographic analysis of maize leaf isoforms to earlier stages of development and found that P-enolpyruvate carboxylase from roots and newly germinated shoots had identical DE52 column elution profiles. These two P-enolpyruvate carboxylase activities also were not inhibited by the addition of leaf P-enolpyruvate carboxylase antiserum to high concentrations which inhibited about 50% of the enzyme activity extracted from leaves. All of these data demonstrate that the

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1 The abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; SDS, sodium dodecyl sulfate.
C4, plant maize has at least two isozymic forms of P-enolpyruvate carboxylase, one of which is used for photosynthetic CO₂ fixation.

We describe the isolation of cloned cDNA probes for P-enolpyruvate carboxylase mRNA and show that P-enolpyruvate carboxylase isozymic forms are encoded by a small gene family. We use a cloned cDNA probe to identify different P-enolpyruvate carboxylase mRNAs present in roots and leaves which encode different isozymic forms.

MATERIALS AND METHODS

Plant Material—An inbred line of maize (B 73, Pioneer Hi-Bred International, Johnston, IA) was used in all studies. For experiments involving leaves and leaf shoots, seeds were germinated and grown as previously described (34). For preparation of root tissue, seeds were spread evenly on heating pads on covered sand benches. Seeds were germinated and grown at 30 °C and kept moist by an overhead sprinkler system.

Protein Purification and Antibody Preparation—P-enolpyruvate carboxylase was purified from green, 3-day-old maize leaves according to the method of Hague and Sims (20). P-enolpyruvate carboxylase activity was measured by monitoring the decrease in activity was measured by monitoring the decrease in

4 °C, after which the supernatant was brought to a concentration of

of the method of Lowry (22).

Preparation of Polyadenylated mRNA—Poly(A) RNA was isolated from seedlings that had been germinated and grown in the dark for 7 days and then transferred to constant light for 5 min before and constant light for 5 min before and constant light for 5 min.

Preparation of Cloned P-enolpyruvate Carboxylase cDNA—Leaf poly(A) RNA was isolated from seedlings that had been germinated and grown in the dark for 7 days and then transferred to constant light for 48 h. Approximately 200 μg of the RNA was denatured in 50% (v/v) dimethyl sulfoxide and layered onto a 36-ml 15-30% (w/v) sucrose gradient, prepared by centrifugation for 30 min at 4 °C, after which the supernatant was brought to a concentration of

P-enolpyruvate carboxylase antisera was prepared by subcutaneously injecting 1 mg of purified P-enolpyruvate carboxylase in Freund's complete adjuvant into a white female New Zealand rabbit. One month following the initial injection, a booster injection of 100-200 μg was given. Total serum was collected 2 weeks later. All protein determinations were conducted using a modification of the method of Lowry (22).

Protein Gel Electrophoresis and Blotting—Leaf or root tissue was ground to a powder in liquid nitrogen, followed by further grinding in 100 mM Tris-HCl, pH 7.5, 2% 2-mercaptoethanol, and 24% (v/v) sucrose. The homogenate was centrifuged at 15,000 × g for 30 min at 4 °C, after which the supernatant was brought to a concentration of

3% (w/v) SDS, 50 mM Tris-HCl, pH 7.5, 2% 2-mercaptoethanol, and 50% (v/v) dimethyl sulfoxide and layered onto a 36-ml 15-30% (w/v) sucrose gradient which was then centrifuged in a Sorvall AH-627 rotor at 24,000 rpm for 18 h at 4 °C. One-ml fractions were collected, and subjected to electrophoresis at 10

mM Tris-HCl, pH 7.5, 2% 2-mercaptoethanol, and 24% (w/v) sucrose. The homogenate was centrifuged at 16,500

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FIG. 1. Identification of P-enolpyruvate carboxylase polypeptide subunits. Blots of soluble proteins, which had been electrophoresed on 7.5—15% SDS-polyacrylamide gradient gels, were reacted with P-enolpyruvate carboxylase antisera. Lane a, 5-day dark-grown leaves; lane b, leaves from 7-day dark-grown + 72-h illuminated seedlings; lane c, 3-day roots; lane d, partially purified root P-enolpyruvate carboxylase; lane e, purified leaf P-enolpyruvate carboxylase. The figure is a composite of blots of several different gels.
serum (Fig. 2, lane c), identifying pPC1 as a clone complementary to P-enolpyruvate carboxylase mRNA from green leaves. The two diffuse bands present in lanes a and b were endogenous translation products of the reticulocyte lysate. Some of the other bands shown in lane b are immunoprecipitable (lane c) and may indicate premature translation termination, internal initiation, or proteolytic cleavage of P-enolpyruvate carboxylase polypeptides. Cleavage was unlikely because the same banding pattern was observed when protease inhibitors were included in the translation reaction mixture (data not shown). Considering the high molecular mass of P-enolpyruvate carboxylase mRNA, which cosediments with 26 S mRNA in a sucrose gradient (17), perhaps it was inefficiently translated because of periodic translation termination due to mRNA secondary structures or RNA degradation (cf. Ref. 35).

Another P-enolpyruvate carboxylase cDNA clone, pPC2, was identified by its ability to cross-hybridize to pPC1 and its ability to hybrid select an mRNA whose translation product was immunoprecipitable by P-enolpyruvate carboxylase antiserum (data not shown). pPC2 was used as a hybridization probe because the size of its cDNA insert, 880 base pairs, was larger than that in pPC1, 445 base pairs. The cDNA insert of pPC1 is identical in restriction sites to one-half of the cDNA insert of pPC2 (data not shown).

P-enolpyruvate Carboxylase Gene Expression in Leaves, Shoots, and Roots—Poly(A) RNA isolated from green leaves and roots was fractionated in denaturing gels and blotted. Hybridization of the blot with pPC2 identified a single size class of P-enolpyruvate carboxylase mRNA in leaves and roots, 3.4 kilobases (Fig. 3, left). The concentration of P-enolpyruvate carboxylase mRNA in each organ was quite different, however. From our recalculation of an earlier reported value (19), we estimate that P-enolpyruvate carboxylase mRNA constitutes 0.20% of total poly(A) RNA isolated from green leaves (9 days post-germination). Since 100 times as much poly(A) RNA was loaded into each root lane as was loaded into each leaf lane, we estimate that P-enolpyruvate carboxylase mRNA represents 0.0025% of root poly(A) RNA. Given the sequence difference between the pPC2 probe and the root form of P-enolpyruvate carboxylase mRNA (Fig. 3, right), this may be an underestimate of the amount of P-enolpyruvate carboxylase mRNA in roots.

The relatedness of P-enolpyruvate carboxylase mRNAs in roots, shoots, and green leaves was measured by hybridizing replicate blots of gel-fractionated mRNA to the same pPC2 probe under identical conditions. Post-hybridization washes were performed at increasing temperatures for each replicate blot. An example of one set of blots is presented in Fig. 3 (left), where the pPC2 probe is shown to be less related to root than to leaf P-enolpyruvate carboxylase mRNA. The data from all experiments are compiled in Fig. 3, (right), in which relative band intensities are plotted as a function of post-hybridization wash temperature. There is an abrupt decrease in the amount of pPC2 probe hybridizing to root or shoot RNA between 45 and 50 °C, suggesting that there is a single form of P-enolpyruvate carboxylase mRNA found in either shoots or roots. The melting profile for leaf mRNA, however, is broader, with a Tm of 54 °C. The broad melting profile suggests that there is more than one form of P-enolpyruvate carboxylase mRNA present in leaves, one of which is identical to the pPC2 probe. Because the form of P-enolpyruvate carboxylase mRNA found in shoots is about 100-fold less prevalent than the leaf forms, shoot P-enolpyruvate carboxylase mRNA contributes no more than 1% of the melt profile of leaf P-enolpyruvate carboxylase mRNA, unless its quantity increases dramatically during leaf development. The Tm of hybrids formed between the pPC2 probe and shoot or root mRNA is approximately 47 °C, indicating an average sequence difference of 7% between root or shoot P-enolpyruvate carboxylase mRNA and the leaf forms of the mRNA (36). The different forms of P-enolpyruvate carboxylase mRNA in leaves could be as much as 10–12% different in sequence.

This kind of thermal denaturation analysis has been shown to give accurate quantitative estimates of sequence relatedness of heteroduplex molecules. Whereas immobilization of DNA or RNA has significant effects on rates of hybridization, it does not affect the denaturation process. For example, McKeown et al. (37) estimated the degree of sequence dissimilarity between different Dictyostelium actin genes by measuring the Tm of hydroxylapatite-bound hybrids. DNA sequencing studies (38) confirmed their estimates. Dunsmuir et al. (39) found that petunia leaf mRNAs encoding different forms of the light-harvesting chlorophyll a/b protein would hybridize to a given cDNA clone bound to nitrocellulose. They showed that mRNAs encoding different forms were differentially eluted by increasing temperatures.

From these data, it is not possible to determine whether the P-enolpyruvate carboxylase mRNA present in roots and shoots is also present in leaves. It can be concluded, however, that there are leaf-specific P-enolpyruvate carboxylase mRNAs which are not present in roots and shoots. There appear to be at least three forms of P-enolpyruvate carboxylase mRNA in maize which exhibit significant differences in sequence. One form of present in roots and may also be the same form found in shoots. At least two forms are present at much higher levels in green leaves.

It is important to emphasize that the P-enolpyruvate carboxylase cDNA probe which we have used in our hybridization analyses, pPC2, represents only 25% of the total length of P-enolpyruvate carboxylase mRNA. Until we obtain sequence information, we do not know to what extent we have measured...
sequence divergence in the untranslated or coding regions of P-enolpyruvate carboxylase mRNA. Preliminary analysis of P-enolpyruvate carboxylase clones isolated from a library of genomic maize DNA indicates that pPC2 is homologous to the 3' end of the gene.

P-enolpyruvate Carboxylase Gene Family—Because we can detect at least three unique P-enolpyruvate carboxylase mRNAs, it is probable that they are encoded by different genes. To arrive at an estimate of the number of P-enolpyruvate carboxylase genes, we hybridized pPC2 probe to Southern blots of genomic DNA which had been digested by a variety of restriction enzymes. Following hybridization, duplicate blots were washed at 42 or 60 °C in the hybridization solution. In all lanes of both blots, two prominent bands are evident (Fig. 4). Because these restriction enzymes do not cut within the cDNA insert of pPC2, each band of hybridization may represent a different P-enolpyruvate carboxylase gene. The presence of introns and sequence differences between genes may give rise to restriction sites not found in our probe, thereby making multiple bands correspond to a single gene. Preliminary analysis of a P-enolpyruvate carboxylase genomic clone has identified such a site. An EcoRI site is present in an intron in the 3' region covered by the pPC2 probe, thus giving rise to the two most darkly hybridizing bands in the EcoRI lanes of both Southern blots. We do not expect heterozygosity of any P-enolpyruvate carboxylase genes because our plant material came from a highly inbred line.

The presence of weakly hybridizing bands on the 42 °C washed filter shows that there are genomic sequences with reduced homology to the pPC2 probe. Given that the thermal stability of pPC2 hybridized to root mRNA is lower than it is to leaf mRNA (Fig. 3, right), it is likely that some of the minor bands shown on the 42 °C washed filter (Fig. 4) correspond to the gene(s) encoding non-autotrophic P-enolpyruvate carboxylase. It is also possible that at least one of the weaker hybridizing bands contains a gene encoding a photosynthetic P-enolpyruvate carboxylase.

DISCUSSION

In this study, the different isozymic forms of P-enolpyruvate carboxylase in maize were shown to be encoded by a
small gene family of about three to six members. At least two of these genes encode prevalent, leaf-specific isoforms involved in the photosynthetic fixation of atmospheric CO₂. This conclusion is based on the thermal denaturation pattern of the pPC2 probe hybridized to RNA blots, which suggests that there are at least two different, prevalent P-enolpyruvate carboxylase mRNAs in maize leaves. Whereas it is clear that these leaf mRNAs are significantly different from each other in base sequence, it cannot necessarily be concluded that they encode correspondingly different polypeptides. Preliminary mapping of a cloned maize P-enolpyruvate carboxylase gene suggests that pPC2 corresponds to the 3' 25% of the mRNAs. It is therefore possible that much of the sequence difference between prevalent leaf P-enolpyruvate carboxylase mRNAs lies in their 3'-untranslated region. These results confirm and provide a genetic explanation for earlier reports of multiple leaf P-enolpyruvate carboxylase isoforms with different enzymological and chromatographic properties.

All of these data suggest significant divergence in the genes encoding maize leaf isozymic forms. Because our cDNA probe spans the 3'-untranslated region of its complementary mRNA, it provides a different measure of the evolutionary history of these genes. If one assumes that the 3'-untranslated region of a gene is under very little selective pressure, our data suggest that the duplication of P-enolpyruvate carboxylase genes to give rise to those encoding leaf isoforms was not a recent event. Nevertheless, the genes encoding prevalent leaf isoforms appear to be coordinate regulated. Martineau and Taylor (41) have shown that the onset of accumulation of prevalent leaf P-enolpyruvate carboxylase mRNAs is coincident with the maturation of mesophyll cells.

Our data suggest, but do not prove, that there is a single non-autotrophic isoform of P-enolpyruvate carboxylase in maize. The enzymological, chromatographic, and antigenic properties of the root and shoot isoforms are identical, suggesting a single non-autotrophic form in both organs. P-enolpyruvate carboxylase mRNA from both roots and shoots exhibits identical thermal stabilities with the pPC2 probe, also suggesting a single form. A more complete understanding of the relationship of the root and shoot isoforms will have to await further study. Another topic for further study will be to determine which isoform of P-enolpyruvate carboxylase operates in stomatal guard cells, or whether the guard cell isoform is different from all others so far described.

Analyses of P-enolpyruvate carboxylase gene families will likely provide insight into the evolution of the C₄ pathway. To date, its evolutionary history is obscure. It has been found in a wide range of plants from both monocots and dicots, and there are a number of examples of C₃ and C₄ plants within the same genus (1). The apparent polyphyletic origins of C₄ photosynthesis suggest that different lineages of C₄ plants may have arisen independently in response to similar selective pressures. One of the primary events in the evolution of C₄ plants may have been the development of a regulatory mechanism which generates a novel pattern of P-enolpyruvate carboxylase gene expression. Future studies should elucidate the sequence relatedness between genes encoding photosynthetic and non-autotrophic P-enolpyruvate carboxylase isoforms, especially those which have functional counterparts in C₃ plants (i.e. the form in leaf stomatal guard cells which also fixes atmospheric CO₂).

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