Pyruvate Orthophosphate Dikinase Gene Expression in Developing Wheat Seeds

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ABSTRACT
The amount of pyruvate orthophosphate dikinase (PPDK) (EC 2.7.9.1) protein in wheat (Triticum aestivum L. var Cheyenne) grains was determined at different stages of development by the protein blot method. The variation in PPDK protein with time in developing wheat grains was similar to that of the enzyme's activity reported by Meyer et al. (1982) Plant Physiol 69: 7–10. The variation in levels of PPDK mRNA with seed development was determined by analysis of polypeptides immunoprecipitated with anti-PPDK serum from in vitro translation products of extracted seed RNA. This mRNA variation was similar to that of the in vitro enzyme levels and the correlation is consistent with the regulation of PPDK gene expression by the level of its mRNA.

The highest level of PPDK in developing wheat seeds occurs later than the highest levels of both ribulose 5-phosphate carboxylase (EC 4.1.1.39) and of chlorophyll, which are located in the green pericarp tissue. PPDK was located in both endosperm and pericarp tissue of the seeds. The tissue location and developmental profile of seed PPDK are consistent with a metabolic role of providing phosphoenolpyruvate as a substrate for recapturing respiratory CO₂ in the seed, and possibly for amino acid interconversions during development.

Although there have been extensive studies of the properties of PPDK (5, 19, 23), information about the regulation of synthesis of the enzyme as a function of development or for specific tissues is still scarce and based on enzyme activity rather than amount of protein. We have examined the developmental profile of both PPDK protein and PPDK mRNA levels in wheat grains. Our results are complementary to the PPDK activity profile previously reported by Meyer et al. (18), and suggest that the enzyme level is determined by the level of PPDK mRNA.

MATERIALS AND METHODS

Plant Material. Wheat (Triticum aestivum L. var Cheyenne) was grown in a greenhouse as described earlier (11). Immature wheat seeds were harvested from 3 to 40 d past anthesis, and were then frozen in liquid N₂ and stored at −80°C until used.

Reagents. Radiochemicals were purchased from New England Nuclear, and chemical reagents from Sigma Chemical Co., and from Biorad Laboratories.

Isolation of Total RNA. Total RNA was isolated essentially as described by Greene (11), from wheat heads tagged at anthesis and harvested at defined intervals afterward; 1.2 g of wheat seeds were homogenized in a tissue grinder in 10 ml of extraction buffer containing 0.1 M Tris-HCl (pH 8.0), 5 mM EDTA, 1.25 mg proteinase K, and 1% SDS. After centrifugation of the homogenate at 13,000g, total RNA was isolated by phenol-chloroform extraction, ethanol precipitation, and pelleting by ultracentrifugation through 5.7 M CsCl (10). Total RNA content of seeds was determined by spectrophotometric analysis of ethanol precipitates assuming ε₅₂₀ = 20.

In Vitro Protein Syntheses. Four micrograms total RNA were translated in micrococcal nuclease-treated wheat germ extract in the presence of [³H]leucine (16) or in reticulocyte lysate (New England Nuclear) in the presence of [³⁵S]methionine (20). Total in vitro protein synthesis in the wheat germ extract was determined by measurement of the amount of [³H] in TCA precipitates by scintillation counting.

Immunoprecipitation of In Vitro Translation Products. Fourteen microliters of the solution of in vitro translation products were immunoprecipitated using anti-PPDK serum (2) and pre-washed protein A, according to the method of Kessler (15). After SDS-PAGE, the gel was stained with Coomassie Brilliant Blue and destained. It was then immersed in En'hance (New England Nuclear). The gel was dried in a vacuum, and then radioauto-

Since the discovery of pyruvate orthophosphate dikinase (14), it has been widely associated with C₄ plants which possess Kranz anatomy, or CAM plants. Meyer et al. (17, 18), however, demonstrated PPDK activity in immature cereal grains, most of which are from plants exhibiting C₃ photosynthetic CO₂ fixation.

The wide occurrence of PPDK in C₃ leaves and seeds and similarities in immunoprecipitation, mol wt of the subunit, and regulatory properties of the enzyme from these tissues to those of the enzyme from C₄ plants (2, 3) suggests the presence of gene(s) for PPDK in C₃ plants similar to the gene for PPDK in C₄ plants. The amount of PPDK found in C₃ tissues so far seems to be considerably less than that found in C₄ tissues. The regulation of the amount of PPDK, possibly through control of gene expression, is of interest to understanding control of organ development (as in seeds), the presumed evolution of C₄ plants from C₃ plants (6), and possibly the eventual genetic engineering of C₃ plants to increase productivity.

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2 Abbreviations: PPDK, pyruvate orthophosphate dikinase; PEPC, phosphoenolpyruvate carboxylase; MDH, malic dehydrogenase; RuBPC, ribulose 1,5-bisphosphate carboxylase/oxygenase; DAF, days after flowering.

3 Reference to a company and/or product named by the Department of Agriculture is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.
graphed at -70°C for 16 d. Amount of PPDK synthesis was estimated by densitometric scanning of the exposed x-ray film.

Protein Blot. Protein blot analysis was carried out as described (3). For location of PPDK and RuBPC subunit bands, two layers of cyanogen bromide paper were used to obtain duplicate transfer blots. One was probed with anti-PPDK and the other was probed with anti-RuBPC. Total soluble protein content was determined by the method of Bradford (7), and total Chl content by the method of Arnon (4).

Location of PPDK in Immature Wheat Grains. Fifty immature wheat grains at each of two developmental stages (approximately 12 to 15 and 30 to 33 DAF) were used. Each grain was rapidly dissected with a pair of forceps, and the pericarp tissue, after being peeled, was immediately washed with protein blot extraction buffer in order to remove the white aleurone layer. The pericarp tissue was then further washed and then was frozen in liquid N2. The endosperm tissue was frozen in liquid N2, as soon as the pericarp was peeled. The nature of this experimental procedure precluded obtaining precise quantitative data, but a rough estimate could be made of relative amount of PPDK in pericarp and endosperm tissue in the 12- to 15-d-old seeds.

Frozen tissues (about 1 g) were ground in a mortar with 1 ml of extraction buffer. The homogenate was filtered through nylon cloth. The filtrate was centrifuged at 13,000g and O'C for 20 min. The supernatant was mixed with the SDS sample buffer according to the method described in the accompanying paper (3) and was subjected to SDS-PAGE.

Sufficient amounts of soluble protein (0.5 mg) were loaded to ensure detection of the enzyme which may be only 0.01% of the total soluble protein. For rough quantitation, 0.125 to 0.5 µg of purified PPDK from maize (3) was loaded on adjacent lanes of the same gel.

RESULTS

Developmental Profile of Wheat Seed PPDK Content. Under our growth conditions, Cheyenne wheat seeds reach maximum fresh and dry weight approximately 35 DAF. The amount of PPDK protein in seeds began to increase at about 6 d after anthesis, reached a maximum value between 20 and 25 d, and declined to the initial value between 35 and 40 d past anthesis (Fig. 1). This result is complementary to that of Meyer et al. (18), who reported that the activity of the enzyme increased from barely detectable at 7 d after anthesis to a maximum about 25 d after anthesis. The agreement between our results and those of Meyer et al. suggests that the activity of PPDK in developing seeds is in direct relation to the amount of enzyme present, and not defined by developmentally determined enzyme activation.

In agreement with Meyer et al. (18), we have observed that the increase and subsequent decline in PPDK content occurs later during seed development than does that of seed Chl (Fig. 1). The peak in PPDK content also occurs later in seed development than that of RuBPC. This is seen when autoradiographs of protein blots for the two enzymes are compared as a function of seed development (Fig. 1). The wheat PPDK subunit migrates as a single band in SDS-gels with an apparent subunit mol wt of about 94,000 (2, 3), while the RuBPC large subunit migrates at about 55,000 (1). The profile of RuBPC is similar to that of Chl, consistent with their linked function and similar location in chloroplasts.

Developmental Profile of PPDK mRNA Activity. In order to compare seed content of PPDK with the seed's potential for synthesis of this enzyme, we isolated total RNA from seeds at different times after flowering, and followed PPDK mRNA activity by immunochemical analysis of in vitro translation products. SDS-gel analysis of anti-PPDK immunoprecipitated in vitro synthesis products shows a prominent band at 94,000 D (Fig. 2), similar to that of authentic PPDK subunit (2).

![Fig. 1](image1.png)

**Fig. 1.** Developmental profile of wheat seed PPDK protein and of RuBPC large subunit. Fresh weight, total soluble protein, Chl, the amount of PPDK and of RuBPC large subunit are expressed as amounts per grain. The amounts of PPDK and of RuBPC large subunit were estimated from the intensity of the protein blot autoradiogram using densitometry.

![Fig. 2](image2.png)

**Fig. 2.** PPDK synthesis in vitro using wheat mRNA. The in vitro-translated proteins were analyzed by SDS-PAGE and the PPDK band was identified by immunoprecipitation by antibody against maize PPDK. PPDK subunit is the largest prominent component in the gel and comigrates with authentic maize PPDK (see "Materials and Methods"). The fast migrating band may be an artifact of degradation resulting from the heating step required prior to gel electrophoresis.
The level of PPDK mRNA activity, per μg RNA, begins to rise at about 9 DAF and reaches a maximum at about 24 DAF (Fig. 3). This profile is similar to that of total seed mRNA activity per μg total RNA and to that of the total seed mRNA activity per seed. The change in in vitro PPDK mRNA activity with seed development, including the decline after 25 d, is also similar to the change in seed PPDK content (Fig. 1). This suggests that the enzyme undergoes a relatively rapid rate of metabolic turnover, and that its content in the seed is determined mainly by its rate of synthesis.

Location of PPDK Within the Seed. A previous report (2) that PPDK protein is present in the pericarp tissue of wheat seeds was inconsistent with the conclusion of Meyer et al. (18) that there was no PPDK activity in this tissue. This question was reexamined with the inclusion of a careful washing step to separate aleurone tissue more completely from the pericarp. Both green pericarp tissue and endosperm tissue from seeds 12 to 15 and 30 to 33 DAF contained PPDK. Precise quantitation was not possible in this experiment, but we estimate from densitometry that 90% or more of the PPDK of the 12- to 15-d-old seeds was in the endosperm. The apparent amount of PPDK in the pericarp from the 30 to 33 d seeds may be exaggerated due to the fact that the precise dissection of these seeds is more difficult than with the 12 to 15 d seeds.

The patterns of bands of PPDK subunits obtained by anti-PPDK probing following SDS-PAGE are quite different for the two tissues (Fig. 4A). The pericarp tissue gives a number of bands of lower mol wt than the main 94-kD band seen with endosperm. Whole seeds that were quickly homogenized and extracted also gave multiple bands, so they are not due entirely to breakdown during dissection and washing.

If this pattern of several bands seen with pericarp tissue represents rapid degradation of PPDK, either in vitro or during extraction, it could explain the inconsistency between our finding of PPDK protein in pericarp tissue and the report that PPDK activity is absent from such tissue (18). Since the present result is based on immunoochemical recognition of the enzyme protein.

Fig. 3. Developmental profile of PPDK mRNA activity as measured by in vitro protein synthesis and detection by immunoprecipitation. Curve A, PPDK mRNA activity per μg total wheat seed RNA; curve B, total wheat seed mRNA activity per μg total wheat seed RNA; curve C, total wheat seed protein mRNA activity per seed.

FIG. 4. Presence of PPDK and of RuBPC in endosperm and pericarp tissue. A. PPDK. B. RuBPC (large subunit). RuBPC is located only in the pericarp tissue. The seed extracts were analyzed by protein blot. Lane 1, 0.5 μg of purified maize PPDK standard. Lanes 2 through 7 each received 0.5 mg of soluble protein from wheat seed tissues, but the number of seeds from which this amount of protein was derived varied from about 0.2 to 1.1. In lanes 2 and 4 (pericarp, 12–15 d) the protein was derived from about twice as many seeds as in lanes 3 and 5 (endosperm, 12–15 d), but in lanes 6 (pericarp, 30–31 d) and 7 (endosperm, 12–15 d) the protein was from about the same number of seeds.
rather than enzymic activity, it eliminates the possibility of a false negative result that might occur due to enzyme inactivation prior to or during tissue preparation. In contrast to PPDK, RuBPC large subunit is seen only in the pericarp tissue (Fig. 4B).

**DISCUSSION**

Use of immunoprecipitation techniques has facilitated demonstration that PPDK is more widely distributed in both leaves and seeds of C₄ and C₃ plants than previously realized (3). Physicochemical and enzymic properties appear to be strongly conserved over this distribution. In the present study we have examined developmental aspects of PPDK expression in wheat seeds to gain insight into the nature of control of such expression, and also to seek further clues about the possible metabolic role of this enzyme in seeds. We have observed that in wheat seeds there is a direct correlation between the amount of PPDK protein and the level of PPDK mRNA activity, and that these two parameters vary with seed development in a manner similar to that of PPDK activity as reported by Meyer et al. (18). Therefore, PPDK mRNA activity, PPDK protein, and PPDK enzyme activity all are correlated with time of development. Furthermore, these profiles are correlated with total protein mRNA activity (unlike the case for RuBPC, for example).

These data suggest that PPDK gene expression in the developing wheat seed, as indicated either by enzyme activity or enzyme protein, is controlled by the level of its mRNA. The level of mRNA is determined by the relative rate of transcriptional processing as compared with degradation, but transcriptional control is more frequently encountered (8). Further work will be required to confirm the nature of the limiting control of seed PPDK gene expression, however.

The 94 kD size of PPDK subunit synthesized in vitro from wheat seed mRNA in this study contrasts with the 110 kD size observed by Hague et al. (12) for the in vitro synthesis product of maize leaf mRNA. Those authors suggested that the maize leaf mRNA of PPDK encodes a larger 'transit peptide' which is cleaved during entry into the chloroplasts to give PPDK subunit. A possible basis for this size difference is the fact that wheat seed endosperm tissue is not photosynthetic, so that PPDK in this tissue is not, a priori expected to require transit into chloroplasts. The apparent size difference may result from tissue specific differences in gene regulation. Consistent with such a possible tissue specific difference are the lack of synchrony between the developmental profiles of PPDK and RuBPC in wheat seed and the report by Meyer et al. (18) that keeping wheat ears dark from anthesis onward causes no change in extractable PPDK activity.

In C₄ mesophyll leaf cells PPDK mediates conversion of pyruvate with ATP and Pi to provide the PEP for carboxylation leading to formation of oxaloacetate (13). In wheat seeds, absence of Kranz anatomy, the location of PPDK both in the white endosperm tissue and in the green pericarp, and the different developmental profile for PPDK activity as compared with Chl and RuBPC all suggest that the principal role of PPDK in seeds is not direct participation in photosynthetic carbon fixation leading to sugar formation. The two steps from pyruvate to oxaloacetate may be important in seed metabolism, nevertheless. As discussed by Meyer et al. (18), and in the accompanying paper (3), seed PPDK may function, through the same sequence of reactions, to recapture respiratory CO₂.

In seeds, this may be part of a process for conversion of alanine to glutamate (3). In the absence of conversion of malate to pyruvate and CO₂ with malic enzyme, as occurs on C₃ bundle sheath leaf cells (22), or of glycolysis, there are few other sources of pyruvate in plant cells besides the deamination of alanine. Glycolytic formation of pyruvate, followed by PPDK conversion back to PEP would be a futile cycle, without metabolic value. If the role of seed PPDK is as just discussed, it may be placed among those enzymes that contribute to the synthesis of seed reserve material, for example, glutamate-pyruvate aminotransferase (21), glutamate-oxaloacetate aminotransferase (9), glutamate dehydrogenase (9), ADP-glucose pyrophosphorylase (24), and UDP-glucose pyrophosphorylase (24). The developmental activity profiles reported for these enzymes in cereal seed endosperm tissue are similar to that of total seed mRNA activity, a large fraction of which codes for seed storage proteins (11), and to the profile of PPDK mRNA activity observed in this study. It is possible that the expression of the genes coding for these enzymes is coordinated in the same way as their observed metabolic activities are. The inclusion of seed PPDK in such a coordinated expression would be consistent with all of its observed properties.

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