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ADPglucose Pyrophosphorylase Is Encoded by Different mRNA Transcripts in Leaf and Endosperm of Cereals[1]

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ABSTRACT

Western blots of soluble protein from wheat, rice, and corn showed that ADPglucose pyrophosphorylase subunits have a size of 50 kilodaltons from endosperm tissue and 43 and 46 kilodaltons from leaf. Antisera to ADPglucose pyrophosphorylase precipitated *in vitro* translation products of 73 and 76 kilodaltons when leaf poly(A)$^+$ RNA was used, whereas endosperm mRNA directed the synthesis of 50 and 56 kilodalton polypeptides. To further study the nature of these mRNA species, an ADPglucose pyrophosphorylase cDNA clone from rice endosperm polyadenylated RNA was obtained and used as a hybridization probe. Northern blots showed that ADPglucose pyrophosphorylase mRNA was slightly larger in leaf (2100 bases) than in endosperm tissue (1900 bases). These studies indicated that in cereals there are at least two tissue specific forms of ADPglucose pyrophosphorylase that are encoded by distinct mRNA transcripts. Analysis of genomic DNA by Southern blotting suggested that ADPglucose pyrophosphorylase is encoded by a small gene family.

ADPglucose pyrophosphorylase is a key regulatory enzyme in the starch biosynthetic pathway of plants (15). In storage and leaf tissue ADPglucose is the principle sugar nucleotide substrate of starch synthase. ADPglucose pyrophosphorylase isolated from leaves is allosterically activated by 3-P-glycerate and inhibited by Pi, and this apparently results in the control of starch synthesis *in vivo* (15). On the other hand, the enzyme from seed tissue is considerably less responsive to allosteric effectors, including those mentioned above (15). Because of the difficulty of purifying the ADPglucose pyrophosphorylase activity from seed tissue, it was not clear whether the low allosteric properties of the seed enzyme activity was a reflection of a different tissue-specific enzyme or simply modification possibly by proteolysis of the same protein.

We have cloned ADPglucose pyrophosphorylase mRNA from rice seeds in order to study the genes and transcripts of this enzyme activity in the cereals. Here hybridization and immunological analyses have been used to compare the sizes of the polypeptides and mRNA transcripts from leaf and endosperm tissues of wheat, rice, and maize. These experiments show that there are different tissue-specific forms of this enzyme that are encoded by distinct mRNA transcripts. Furthermore, analysis of the genes suggests that ADPglucose pyrophosphorylase is encoded by a small differentially expressed gene family.

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[2] The order of the first two authors was randomly determined.

MATERIALS AND METHODS

Plant Material. Germinated *Triticum aestivum* L. var Cheyenne was vernalized for 6 weeks at 7°C and grown in a greenhouse. *Oryza sativa* was grown in a controlled environment; *Zea mays* and spinach were grown in a greenhouse. Developing heads and healthy mature leaves were stored at −80°C. Seeds were separated from pericarp and other maternal tissue under liquid N_2 and stored at −80°C.

Isolation of poly(A)$^+$ RNA[3]. The following methods were based on established protocols reviewed by Lizzardi (12). All labware and solutions, where appropriate, were sterilized to eliminate ribonuclease. Each g of seed pulverized under liquid N_2 was suspended in 10 ml of buffer containing 4 M guanidine HCl, 10 mM Tris-HCl (pH 8), 10 mM EGTA, 0.5% (w/v) Sarkosyl, 1% (w/v) insoluble PVP, and 5% β-mercaptoethanol. The suspension was homogenized briefly with a Polytron and extracted with an equal volume of phenol:chloroform. Nucleic acid was precipitated overnight at 0°C by addition of 5 volumes of 4 M LiCl. Poly (A)$^+$ RNA was obtained by olgio(dT)-cellulose chromatography.

***In Vitro* Translation and Immunoprecipitation.** One μg of poly(A)$^+$ RNA and 25 μCi of [^{35}S]methionine (1100 Ci/mmol, NEN) were added per 25 μl wheat germ translation reaction (1). Aliquots containing 1×10^6 hot TCA-precipitable cpm were incubated with spinach leaf ADPglucose pyrophosphorylase antisera and protein A-Sepharose as described (9). Antiserum to spinach leaf ADPglucose pyrophosphorylase was a generous gift from Dr. Jack Preiss (Michigan State University). Immunoglobulin G was purified from the sera by chromatography on protein A-Sepharose (9). Dried protein A-Sepharose-immunoconjugated translation products were suspended in SDS sample buffer for SDS-PAGE (11).

cDNA Library Construction and Screening. The preparation of double-stranded DNA complementary to rice seed poly(A)$^+$ RNA and its ligation to purified λgt11 arms was carried out as described by Huynh *et al.* (8). Packaging efficiency was approximately 3×10^6 pfu/μg of cDNA, with about 10% recombinants as judged by clear plaque formation on enriched media plates containing 5-chloro-4-bromo-3-indolyl β-D-galactoside (X-gal). λgt11 Recombinants were plated on host strain Y1090 at a density of 3×10^4 pfu per 90-mm plate and plaques were screened with purified anti-ADPglucose pyrophosphorylase as described (8, 17).

Isolation of High Molecular Weight DNA. Nuclei were isolated by ethidium bromide stabilization (10), then lysed with 1% Sarkosyl, 50 mM EDTA, and 100 μg/ml proteinase K. The nuclear digest was brought to a density of 1.5 g/ml by adding saturated CsCl, and the DNA was banded overnight in a vertical rotor at 200,000*g*. The band was carefully collected, ethidium

[3] Abbreviations: poly(A)$^+$ RNA, polyadenylated RNA; poly(A), polyadenylic acid.

bromide was removed by butanol extractions, and the DNA was exhaustively dialyzed against 10 mM Tris (pH 7.5), 1 mM EDTA.

Southern and Northern Blot Hybridizations. DNA was digested with restriction enzymes and then concentrated by ethanol precipitation (14). Digested DNA (10 μg) was run on a 0.5% agarose gel alongside gene copy number standards. Poly(A)$^+$ RNA (1–2 μg) was resolved on 1% agarose-formaldehyde or glyoxal gels (14). Both types of gel were transferred by capillary blot to GeneScreen$^+$ (New England Nuclear) and hybridized according to the manufacturer's recommendations to cDNA inserts radioactively labeled by nick translation (14). The hybridization buffer contained 50% formamide, 600 mM NaCl, 120 mM Tris, 0.1% SDS, 0.02% BSA, 0.02% PVP, 0.02% Ficoll, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA, and 10 μg/ml poly(A). The stringency of hybridization was approximately 15°C below Tm.

Western Blotting. Soluble proteins were extracted according to the protocol of Galili and Feldman (7) and total seed proteins were extracted with SDS sample buffer (11). Western blotting to nitrocellulose sheets was essentially as described (3). The sheets were incubated in heat-sealable bags with 36 μg/ml purified immunoglobulin G and then washed with Tris-saline. Then 1 μCi of ^{125}I-labeled *Staphylococcus aureus* protein A (New England Nuclear) was added and incubation continued for 30 min. Immunoreactive bands were visualized by autoradiography using a Cronex (DuPont) intensifying screen.

RESULTS

Sizes of the Nascent and Mature Polypeptides. Because of the different enzymological properties of ADPglucose pyrophosphorylase from leaf and storage tissues, we first investigated the sizes of the enzyme subunits from these tissues using the Western blot technique (Fig. 1). In a previous study purified spinach leaf ADPglucose pyrophosphorylase revealed two size forms of 48 and 44 kD (4) and Western blot analysis confirms this earlier observation (Fig. 1, lane 1). Protein from the leaf tissue of wheat (lane 2), rice (lane 4), and maize (lane 6) also contained two immunoreactive polypeptides of 43 and 46 kD, albeit slightly smaller than the two spinach leaf enzyme subunits (4). The larger immunoreactive band from wheat leaf did not resolve in a discrete band due to the presence of abundant levels of the large subunit of ribulose bisphosphate carboxylase on these polyacrylamide gels. Because of the different enzymic properties exhibited by the seed ADPglucose pyrophosphorylase, protein fractions of endosperm tissue from the same plants were examined. In contrast to the leaf tissue, the endosperm protein from all three cereals contained a single predominant polypeptide of 50 kD that was immunoreactive with the spinach leaf antibody. In a few instances, especially for the wheat sample, a faint lower molecular band was observed which may be a product of proteolysis. The estimate of 50 kD is consistent with the molecular size of purified maize endosperm ADPglucose pyrophosphorylase (J Preiss, personal communication). Therefore, results described here, as well as others, indicate that the leaf and seed forms of ADPglucose pyrophosphorylase differ in the number and size of their subunits.

Nuclear-encoded chloroplast proteins contain a transit peptide to facilitate their transport into this organelle (5). To determine whether the ADPglucose pyrophosphorylase subunits were synthesized as larger precursors, poly(A)$^+$ RNA isolated from leaf and endosperm tissues of wheat was translated in a wheat germ cell-free system and the products were immunoprecipitated and resolved by SDS-PAGE (Fig. 2). As can be seen in Figure 2, wheat leaf poly(A)$^+$ RNA directed the synthesis of two polypeptides with sizes of 73 and 76 kD, considerably larger than the mature polypeptides. These results are consistent with the view that the leaf enzyme is synthesized with an additional leader

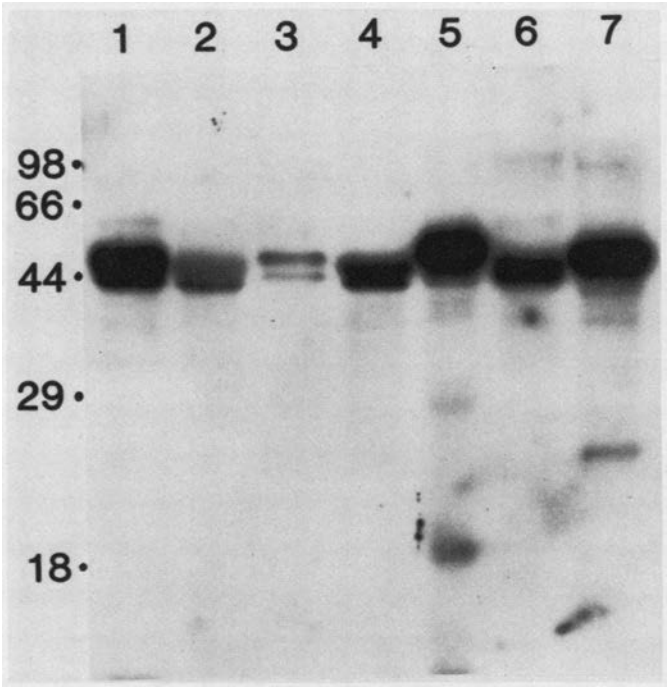

FIG. 1. Western blot analysis of leaf and endosperm ADPglucose pyrophosphorylase. Soluble protein (50 μg) was resolved on a 12% SDS polyacrylamide gel, transferred to nitrocellulose, and probed with ADPglucose pyrophosphorylase antisera and ^{125}I-labeled protein A as described in "Materials and Methods." The source of protein for each lane is: (1) spinach leaf, (2) wheat leaf, (3) wheat seed, (4) rice leaf, (5) rice seed, (6) maize leaf, (7) maize seed. Molecular size markers are indicated on the left.

sequence of about 30 kD. In contrast, wheat endosperm poly(A)$^+$ RNA produced two *in vitro* translated products, one with the mature size of 50 kD, and the other having a size of 56 kD. Similar results were obtained when these mRNA samples were translated in a reticulocyte lysate protein synthesizing system. Therefore it is not clear whether the 50 kD polypeptide represents a processed form or is a product of proteolysis. These results, however, are consistent with the notion that tissue-specific mRNA transcripts account for the different leaf and seed ADPglucose pyrophosphorylase polypeptides.

Cloning of ADPglucose Pyrophosphorylase mRNA. To further study the mRNA that encodes these tissue-specific isozymes, a cDNA clone for ADPglucose pyrophosphorylase was obtained. The strategy involved constructing a rice seed cDNA library in the λgt11 expression vector, followed by screening for antigen-producing clones using established protocols (8, 17). Of six positive clones selected on the first screening, three were true positives on successive screenings. DNA from these clones was prepared in quantity, cut with EcoR1, and checked for insert size. The clone that had reacted most strongly to the antibody during screening (clone AGP-1) had the largest insert size of 1.7 kilobases. The insert from this clone was labeled with ^{32}P by nick translation and used to probe a Northern blot of poly(A)$^+$ RNA from seed and leaf tissue of wheat and rice (Fig. 3). This probe revealed messenger RNA bands of about 1.9 and 2.1 kilobases from seed and leaf tissue, respectively, indicating that clone AGP-1 was nearly full length. Furthermore, the mRNAs from leaf and seed had different sizes that reflected the different sizes of the translation products they presumably encoded.

Organization of ADPglucose Pyrophosphorylase Genes. The organization of the genes for ADPglucose pyrophosphorylase was determined using Southern blotting (Fig. 4). Rice leaf DNA was digested with several restriction enzymes and probed with

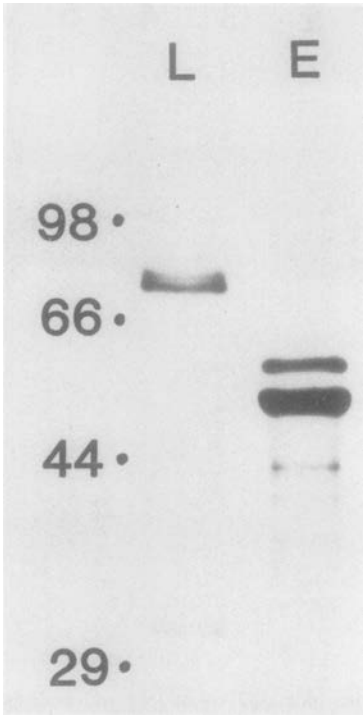

FIG. 2. ADPglucose pyrophosphorylase *in vitro* translation products from wheat. Poly(A)$^+$ RNA from leaf (L) and endosperm (E) was translated in a wheat germ cell-free system and the labeled translation products were purified by protein A-Sepharose affinity chromatography and resolved on a 12% SDS polyacrylamide gel. Size markers are indicated at the left.

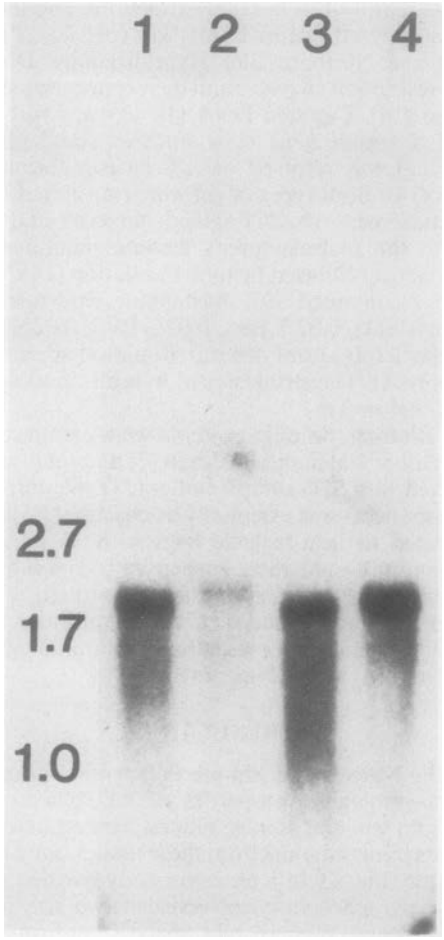

FIG. 3. Northern blot analysis of leaf and seed mRNA. One μg of seed or 5 μg of leaf poly(A)$^+$ RNA was glyoxylated and resolved on a 1% agarose gel using glyoxylated EcoRI/ScaI fragments of pUC19 as markers. The gel was blotted to GeneScreen$^+$ followed by hybridization to the nick-translated insert of ADPglucose pyrophosphorylase cDNA clone AGP-1. Source of the poly(A)$^+$ RNA for each lane was: (1) wheat seed, (2) wheat leaf, (3) rice seed, and (4) rice leaf. Position of the markers are indicated at the left.

clone AGP-1 labeled by nick translation. Amounts of clone AGP-1 equivalent to various copies of the gene were run alongside the rice DNA fragments. With all three enzymes, two or three bands were seen in the size range of 3 to 5 kilobases and there were a total of about three gene copies per haploid genome. Thus, ADPglucose pyrophosphorylase genes are organized in a small family that can be divided into at least two groups on the basis of distinct restriction fragments. This is supported by our preliminary physical mapping studies of the seed cDNA and a recently isolated near full length leaf cDNA. The seed cDNA contains a single HindIII site whereas both the leaf and seed forms lack restriction sites for BamHI and EcoRI (HB Krishnan, CD Reeves, TW Okita, unpublished observations).

DISCUSSION

We have shown that ADPglucose pyrophosphorylases of cereals are present as different tissue-specific isozymes in leaf and endosperm tissues. This is consistent with previous enzymological studies that demonstrated different allosteric properties for ADPglucose pyrophosphorylases isolated from leaf and storage tissues (15). We have further shown that these isozymes are encoded by different mRNA transcripts and do not result from differential posttranslational processing.

The subunit size we observed for the cereal endosperm enzymes agreed with the size previously reported for the potato tuber ADPglucose pyrophosphorylase (16) and the purified maize endosperm enzyme (J Preiss, personal communication), but disagrees with another reported size of 96 kD for the maize endosperm enzyme subunits (6). The enzyme from cereal leaves appeared to be composed of two distinct subunits as is the enzyme from spinach leaves (4), although the cereal subunits were slightly smaller than those detected in spinach leaves. Since immunological cross-reactivity has been observed between ADPglucose pyrophosphorylase of spinach and *E. coli* (J Preiss, personal communication), it is not surprising that the molecular size and antigenic epitopes of the enzymes have been evolutionarily conserved between monocot and dicot plants. Although tissue-specific posttranslational modifications could account for the differences in the sizes of the leaf and seed forms, extensive modification, such as core glycosylation, has not been reported for any plastid-localized proteins. Inasmuch as other forms of protein modification, e.g. phosphorylation, would have only a minor influence on the mobility of proteins on SDS polyacrylamide gels, the size estimates of the ADPglucose pyrophosphorylase polypeptides determined here are likely to be a direct reflection of the lengths of their primary sequences.

ADPglucose pyrophosphorylase has been shown to be localized in the chloroplast (15) or amyloplast (13) and, therefore, the enzyme subunits are expected to be synthesized as precursors with transit sequences to facilitate transport into the plastid and processing to the mature form (5). Wheat leaf poly(A)$^+$ RNA directed the synthesis of two polypeptides with sizes of 73 and 76 kD that are apparently precursors to the 43 and 46 kD subunits. Endosperm poly(A)$^+$ RNA directed the synthesis of a 56 kD polypeptide that presumably represents a precursor with a 6 kD leader sequence. A mature-sized polypeptide (50 kD) that

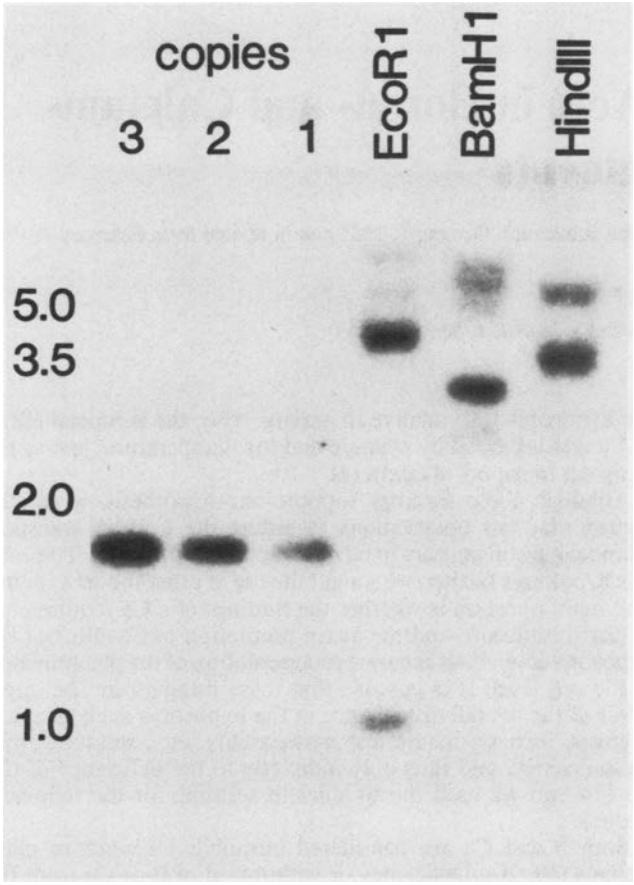

FIG. 4. Organization of rice ADPglucose pyrophosphorylase genes. Ten μg of rice genomic DNA was digested with EcoRI, HindIII, or BamHI and resolved along with ADPglucose pyrophosphorylase cDNA copy number standards on a 0.8% agarose gel. The gel was blotted to GeneScreen+ followed by hybridization to nick-translation-labeled AGP-1 insert. Size markers are indicated at the left.

presumably resulted from partial processing in the wheat germ extract and reticulocyte lysate was also seen, but the ratio of the putative mature and precursor forms was not consistent between experiments. Recently, using wheat endosperm mRNA, a mature-sized *in vitro* translation product was observed for the normally chloroplast-localized enzyme, pyruvate Pi dikinase (2), and the authors concluded that this enzyme is not transported to the amyloplast, but instead functions in the cytoplasm (2). In contrast to the speculations suggested by this earlier study (2), the location of the ADPglucose pyrophosphorylase in amyloplasts is well documented. Therefore, it is not clear whether a transit leader sequence is required for import of ADPgluclose pyrophosphorylase in the amyloplast or whether their *in vitro* translated subunits are particularly prone to proteolytic activity present in these protein synthesizing extracts. Further studies are clearly needed to resolve this problem.

Based on the difference in the estimated molecular sizes of the mature and precursor forms, the leaf ADPglucose pyrophosphorylase apparently has a leader sequence of about 30 kD. This is considerably larger than the 4 to 6 kD transit sequences reported for most nuclear-encoded, chloroplast-localized proteins (5). Possibly portions of the long leader sequence of ADPglucose pyrophosphorylase are required for assembly of the tetramer within the stroma or for maintaining solubility until the tetramer has formed.

Analysis of genomic DNA indicated that there are approximately three copies of the ADPglucose pyrophosphorylase gene per haploid rice genome and that these genes divide into at least two groups based on different sized restriction fragments. This is further supported by the different restriction enzyme sites observed for leaf and seed cDNAs (HB Krishnan, CD Reeves, TW Okita, unpublished observations). The gene copy number may be an underestimate if the leaf and seed and mRNA transcript sequences have diverged significantly. In any event, based on evidence presented here it seems probable that these two groups are composed of differentially expressed members of a small gene family and that the groups encode the leaf and endosperm mRNA transcripts that we have observed. The sequencing of tissue-specific cDNA and genomic clones for ADPglucose pyrophosphorylase will allow this notion to be studied further.

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