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Identification and Molecular Characterization of *Shrunken-2* cDNA Clones of Maize

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Mutation at the *shrunken-2* (*Sh2*) locus of maize, a gene described more than 40 years ago, greatly reduces starch levels in the endosperm through its effect on the starch synthetic enzyme ADP-glucose pyrophosphorylase, an enzyme thought to be regulatory in this biosynthetic pathway. Although our previous work has suggested that *Sh2* is a structural gene for this enzyme, we have also reported data compatible with *Sh2* acting post-transcriptionally. In this study, we took advantage of a transposable element-induced *Sh2* allele, its progenitor, and revertants to identify a clone for this locus. Although the cloning and identification were done independently of any knowledge concerning the product of this gene, examination of the deduced amino acid sequence revealed much similarity to known ADP-glucose pyrophosphorylase subunits of plants and bacteria, including regions involved in substrate binding and activator binding. Little sequence similarity, however, was found at the DNA level. These observations provide direct evidence that *Sh2* encodes a subunit for endosperm ADP-glucose pyrophosphorylase. Analysis of several phenotypically wild-type alleles arising from a mutable *sh2-Ds* allele revealed one unexpected case in which DNA sequences of *Sh2* were rearranged in comparison with the progenitor *Sh2*. In contrast to wild type, the *Ds*-induced *sh2* allele conditions at least two transcripts in the endosperm.

INTRODUCTION

One class of the many genes in maize has been described that greatly alters starch content of the seed. Mutation at either of two unlinked loci, *shrunken-2* (*Sh2*) or *brittle-2* (*Bt2*), gives rise to a severely shrunken, brittle seed. The classical studies of Tsai and Nelson (1966) revealed that both loci affect the starch synthetic enzyme ADP-glucose pyrophosphorylase. This enzyme (ATP: α-D-glucose-1-phosphate adenyltransferase EC 2.7.7.27) catalyzes the reversible synthesis of ADP-glucose and pyrophosphate from ATP and glucose 1-phosphate. It may represent one of the main regulatory steps in the biosynthesis of starch in plants (Preiss, 1982) and glycogen in bacteria (Preiss, 1984). Starch composes approximately 70% of the dry weight of the maize kernel and is, therefore, the major component of the seed weight.

In our earlier studies to determine the mechanism by which *Sh2* and *Bt2* control this enzyme, characterization of the endosperm enzyme from wild-type and several *sh2* and *bt2* mutants revealed that each enzyme preparation had an allele-specific K_m value for glucose 1-phosphate, and that the level of enzyme activity was *Sh2* and *Bt2* dosage dependent (Hannah and Nelson, 1975, 1976). These data suggested that both loci were structural genes for endosperm ADP-glucose pyrophosphorylase. However, in another set of experiments (Hannah et al., 1980), two differentially heat-labile forms of ADP-glucose pyrophosphorylase were detected. All *sh2* and *bt2* mutants analyzed greatly reduced or abolished the major heat-labile form of the enzyme but only moderately reduced the minor heat-stable form of the enzyme. These results raised the possibility that *Sh2* and/or *Bt2* might act post-transcriptionally or post-translationally. In this model, the heat-stable enzyme would be an intermediate in the formation of the heat-labile enzyme. Conversion of an enzymically inactive protein to the heat-labile form through the heat-stable form would require, at each step, action of a protein controlled by *Sh2* and *Bt2*. This could explain the preferential effects of *sh2* and *bt2* mutants on the heat-labile enzyme because formation of the heat-labile enzyme would require two conversion steps, whereas formation of the heat-stable enzyme would require only one conversion. Although this hypothesis explained the data at the time, it was not consistent with later experiments involving kinetic parameters of the two enzymes. The K_m values of the heat-labile and heat-stable forms of the enzyme were found to be identical in the wild type. Furthermore, in the *Sh2* revertants obtained by excision of the transposable element *Ds*, the enzyme levels were approximately equal

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to wild type, but various kinetic parameters were altered (Tuschall and Hannah, 1982). Neither of these two results was consistent with the post-transcriptional role for *Sh2* or *Bt2* in controlling the enzyme action, and the results strongly supported the idea of the two genes being structural genes for the enzyme. However, these somewhat complicated and possibly conflicting observations concerning the nature of genetic control of ADP-glucose pyrophosphorylase by the *Sh2* and *Bt2* genes necessitated more direct molecular studies.

The ADP-glucose pyrophosphorylase from *Escherichia coli* is a homotetramer of subunits of M_r 50,000 (Haugen et al., 1976) and is encoded by a single gene (Baecker et al., 1983). The plant enzymes, on the other hand, have been reported to be a homotetramer in potato (Sowokinos and Preiss, 1982) or heterotetramers, with subunits belonging to two different size classes, in spinach (Morrell et al., 1987a, 1987b), wheat, rice (Krishnan et al., 1986), and *Arabidopsis* (Lin et al., 1988a). Corn endosperm enzyme was earlier reported to be a homotetramer of 54 kD subunits (Plaxton and Preiss, 1987), but a second peptide of 60 kD is now known to be associated with it (C. Barton, *unpublished observations*). Immunological properties and amino-terminal sequences of the two subunits of spinach enzyme differ (Morrell et al., 1987a, 1987b), and it has been suggested that the two different size classes of the ADP-glucose pyrophosphorylase polypeptides are products of two separate genes (Morrell et al., 1988). The enzyme in *Arabidopsis* is also under the control of at least two genes, one of which is possibly regulatory (Lin et al., 1988a, 1988b).

RESULTS

Proof that the 1.3-kb Clone, pES6-66, Is *Sh2*

The 1.3-kb cDNA clone pES6-66, isolated earlier by Carolyn Barton, was used to isolate the *Sh2* clones in this study. Briefly, pES6-66 was isolated from a cDNA library prepared from "Mainliner" endosperms harvested 21 days post-pollination with cloning at the PstI site of pBR322. Candidate *Sh2* clones were identified by differential screening with labeled cDNA of near isogenic W64 *Sh2* and W64 *sh2* mRNA. pES6-66 hybridized to wild-type cDNA and not *sh2* cDNA. pES6-66 was subsequently shown to hybridize to a restriction fragment length polymorphism associated with normal *Sh2* function in F2 segregants of *Sh2* × *sh2* crosses in three backgrounds (Barton et al., 1986; C. Barton, unpublished results). To determine whether this clone was indeed *Sh2* or simply a closely linked gene, we took advantage of a series of maize stocks that differed only at the *Sh2* locus. These stocks, described in detail earlier (Hannah et al., 1980), differed by the presence or absence of the transposable element *dissociation* (*Ds*) in *sh2*. The progenitor stock refers to a wild-type *Sh2* allele contained within a McClintock line that also contained the autonomous element *activator* (*Ac*) and the *Ac*-responding *a1* mutable allele *a1-m3*. From a cross of this stock to an *sh2* tester, one *Ac*-responding mutable *sh2*, termed *sh2-m1*, was found. From self-pollinations of a stock homozygous for *sh2-m1* and containing *Ac*, 44 revertant *Sh2* alleles were isolated. All stocks, when isolated, contained the original allele *a1-m3* at the closely linked (*0.2 map* units) *a* locus. All revertants described were homozygous, and the *sh2-m1* stock analyzed here lacked *Ac*.

EcoRI, XbaI, and SstI digests of *sh2-m1*, its progenitor, and several revertants were probed initially with the 500-bp HindIII-PstI fragment of 1.3-kb cDNA clone pES6-66. Figure 1 shows that the digestion pattern of DNA from *sh2-m1* differs from those of the other genotypes. Digestion with EcoRI or XbaI yielded a fragment in *sh2-m1* that is approximately 1600 bp larger than that seen in the progenitor or revertant R1. Furthermore, SstI digest yielded two hybridizing bands in *sh2-m1* but only one band in the progenitor. The total size of the two SstI fragments is approximately 1600 bp larger than the single SstI fragment hybridizing in the progenitor stock. The data are compatible with *sh2-m1* containing a 1600-bp insertion and the insertion containing an internal SstI site.

Figure 1 also contains genomic digests of the revertant *Rev5*. The hybridizing pattern of this revertant differs from that of Rev1, the progenitor, and the mutable allele. DNA gel blot analysis of 22 other revertants (data not shown)

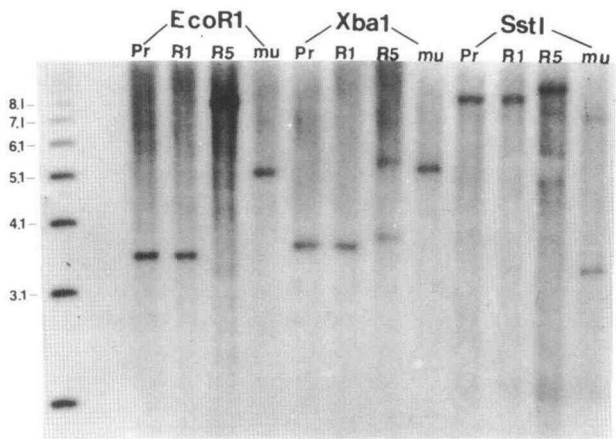

Figure 1. Restriction Fragment Length Polymorphism between Progenitor Allele of *Sh2* (Pr), Its Mutant (mu), and Two Phenotypically Wild-Type Revertants (R1 and R5).

Genomic DNA gel blots of EcoRI, XbaI, and SstI digests of DNAs isolated from above-mentioned stocks were probed with a 500-bp HindIII-PstI fragment of the 1.3-kb cDNA clone, pES6-66 (Barton et al., 1986). The 1-kb DNA ladder (Bethesda Research Laboratories) was used as molecular size standard.

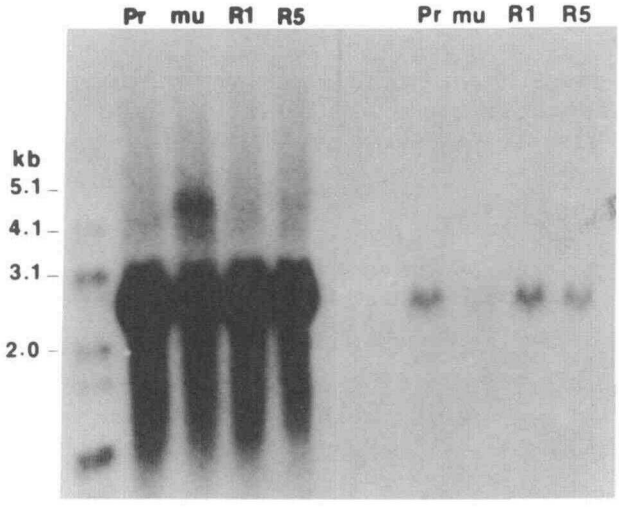

Figure 2. RNA Gel Blot of Total RNA Extracted from 22-Day-Old Seeds of Progenitor Allele (Pr), the Mutant (mu), and Revertants (R1 and R5).

The probe and the molecular size standard used were the same as those in Figure 1. The blot was exposed for two different time periods: for correct estimate of size of the transcripts (short exposure, right half) and to show the second larger transcript in the mutant allele (long exposure, left half). Equal amounts of RNA were loaded on the gel.

yielded patterns that were nearly identical to that observed for *Rev1*. Thus, *Rev5* is unique among all of the *sh2-m1* revertants; we are not aware of any *Ds* revertants of other mutable alleles that give rise to such complicated DNA rearrangements. We are investigating the allele further.

Because the 1.3-kb cDNA clone detected a DNA polymorphism that distinguishes *sh2-m1* from its progenitor and its revertants, we conclude that this clone is not a gene closely linked to *Sh2* but is, in fact, *Sh2*.

Further proof of the identity of the clone was obtained in RNA gel blots of the poly A^+ RNA of the progenitor, mutable, and revertant alleles. The results are depicted in Figure 2. The probe detected a single transcript of about 2.0 kb to 2.2 kb in the progenitor and all the revertants, although it detected two transcripts in *sh2-m1*. Because five of 11 spontaneous *sh2* mutants (data not shown) totally lacked a transcript at the wild-type position, we conclude that the faster migrating transcript in *sh2-m1* is under the control of the *Sh2* locus. Because of the presence of the second transcript in *sh2-m1*, it is interesting to consider the recent evidence (reviewed by Wessler, 1988) showing that transposable elements can sometimes be removed from transcripts by mechanisms normally thought to be involved in intron splicing. This might suggest that the larger transcript in *sh2-m1* represents the transcript before *Ds* removal, whereas the one of wild-type size would occur after *Ds* excision. In such cases, one would predict that the increase in the size of the transcript would be equal to the size of the insertion. We observed, however, that the larger transcript, as sized on RNA gels, was some 2800 nucleotides larger than the wild-type transcript and the observed increase of the DNA was only 1600 bp. We are investigating the origin of the larger transcript of *sh2-m1*.

Isolation of Maize *Sh2* cDNAs

The 500-bp fragment described above was used to screen the cDNA library constructed from poly A^+ RNA of *shrunken-1*, *bronze-mutable4* (*sh1 bz-m4*). Fourteen clones were selected at random from a group of approximately 50 hybridizing clones. EcoRI digests were probed with the two end fragments of the pES6-66, i.e., the 500-bp HindIII-PstI fragment used in Figure 1 and a 250-bp PstI-EcoRI fragment, to isolate the largest cDNA clone. The largest fragment hybridizing to the 500-bp probe was approximately 1050 bp, whereas the fragment hybridizing to the 250-bp probe was about 900 bp (data not shown). Thus, the cDNA insert of the largest clone hybridizing to both the 5′ and 3′ ends of pES6-66 was about 1.95 kb, with an internal EcoRI site at about 900 bp from the 5′ end, as indicated in Figure 3. RNA gel blot analysis (Figure 2) indicated that the size of the wild-type transcript is approximately 2.0 kb to 2.2 kb. Eight of the 14 clones isolated had the 1.95-kb insert and were almost full length. The 900-bp and 1050-bp EcoRI fragments from one of these clones were subcloned into pUC119 (referred to as

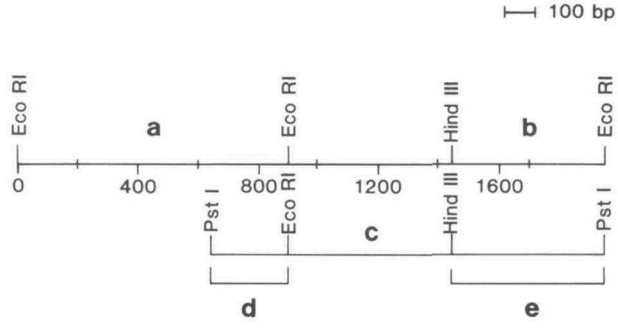

Figure 3. Orientation of the cDNA Clones.

(a) and **(b)** EcoRI fragments pc*Sh2*-1a and pc*Sh2*-1b, respectively, of the 1.95-kb *Sh2* cDNA clone isolated from the *sh bz-m4* cDNA library in the present studies.
(c) pES6-66 isolated earlier (Barton et al., 1986).
(d) and **(e)** Probes used for hybridization with the EcoRI digests of the 14 positive clones.
(e) Probe used in Figures 1 and 2.

pc*Sh2*-1a and pc*Sh2*-1b, respectively), then into the M13mp19 vector, and sequenced. The 800-bp PstI-HindIII fragment of pES6-66 was also sequenced.

Primary Structure of the cDNA Clones

The nucleotide sequence of pc*Sh2*-1a and pc*Sh2*-1b is given in Figure 4 and represents the 5′ to 3′ sense strand of the *Sh2* cDNA. The 5′ to 3′ direction is based on the presence of a poly A tail on pc*Sh2*-1b. The sequence of the 800-bp fragment of pES6-66, which flanks both pc*Sh2*-1a and pc*Sh2*-1b, was used to confirm the 5′ to 3′ direction of pc*Sh2*-1a. It also allowed us to conclude that there was only one EcoRI site separating pc*Sh2*-1a and pc*Sh2*-1b. The 800-bp clone had the EcoRI site at about 250 bp from the 5′ end, and sequences on either side of it were identical to pc*Sh2*-1a or pc*Sh2*-1b, thus confirming the orientation of the 1.95-kb *Sh2* cDNA (Figure 3). The insert of pc*Sh2*-1a was 891 bp and that of pc*Sh2*-1b was 1030 bp followed by several A residues. The cDNA sequence was analyzed for all restriction sites. Some of the major restriction sites are shown in Figure 4. The positions of the HindIII, ClaI, BglII, SphI, XhoI, and AvaI sites were also confirmed by restriction digestions of the 900-bp and 1050-bp EcoRI inserts isolated from plasmids (data not shown).

Consensus Sequences of the cDNA

No typical eukaryotic poly A^+ addition signal (AAUAAA) (Proudfoot, 1984) was found after the stop codon. Variations of this sequence, AACAAA and ACTAAA, were found within the usual 10 bp to 30 bp of the poly A site (Figure 4) and may serve as the polyadenylation signal. Other multiple atypical poly A^+ addition signals have been reported in certain maize genes (Furtek et al., 1988; Ralston et al., 1988).

Derived Amino Acid Sequence of the *Sh2* cDNA Sequence Has Similarity to ADP-Glucose Pyrophosphorylase

Examination of the 1.95-kb cDNA sequence identified only one open reading frame (ORF) coding for 542 amino acids and making up a polypeptide of 59,845 Da. As indicated in Figure 5, the first methionine in the long open reading frame was amino acid 27; however, the sequence 5′ to it also represents an ORF, and our cDNA may not be complete at the 5′ end, as judged by the RNA gel blot data (Figure 2). The derived amino acid sequence of the ORF of the 1.95-kb *Sh2* cDNA was compared with approximately 7000 known sequences in the National Biomedical Research Foundation-Protein Identification Resource protein data bank, and the *E. coli* glucose-1-phosphate adenyltransferase (ADP-glucose pyrophosphorylase or ADP-glucose synthetase, EC 2.7.7.27) was the only protein found to have significant similarity to the amino acid sequence of the *Sh2* cDNA. The amino acid sequence of ADP-glucose pyrophosphorylase of *E. coli* was derived

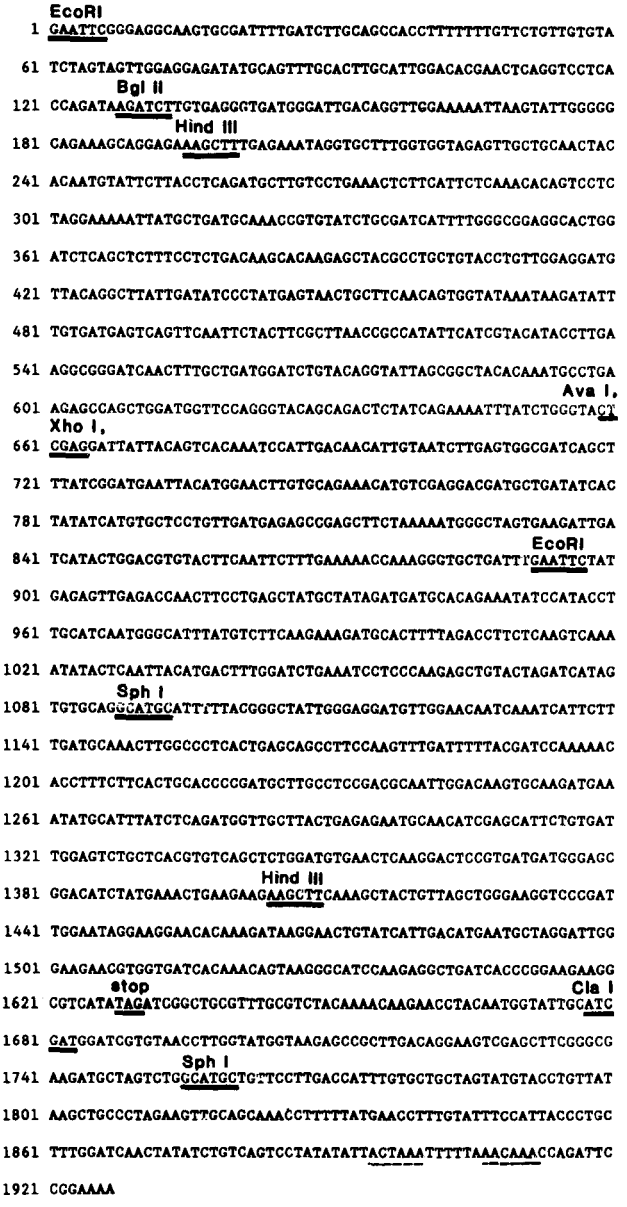

Figure 4. Nucleotide Sequence of the 5′ to 3′ (Left to Right) Sense Strand of the 1.95-kb *Sh2* cDNA Clone.

The major restriction sites and the stop codon are underlined and the putative poly A^+ signal sequences are indicated by broken underlines.

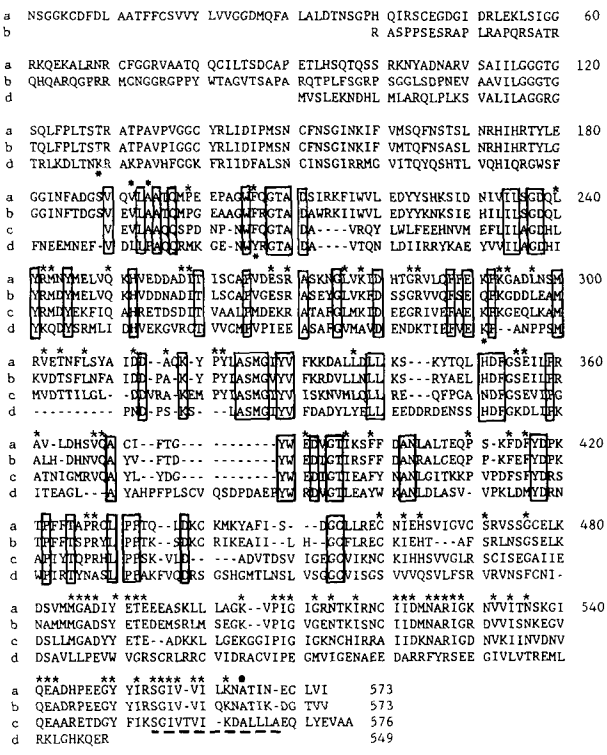

Figure 5. Comparison of the Derived Amino Acid Sequences of ADP-Glucose Pyrophosphorylase cDNAs.

(a) Maize endosperm (present work).
(b) Wheat endosperm WE:AGA.7 (Olive et al., 1989).
(c) Rice endosperm (Morrell et al., 1987a).
(d) *E. coli glgC* gene (Baecker et al., 1983).
Amino acids identical to all four species in the overlapping reading frames are indicated by enclosed boxes. Amino acids shared by only the three plant species are indicated by an asterisk (*) over the lines. Lysine residues 129 and 291 and tyrosine 206 implicated in the active sites of the enzyme (see Results) are marked by an asterisk (*) under the residues. The underlined residues 554 to 568 are compared with the related sequence of spinach enzyme in Figure 6. Gaps were introduced to maximize identities, and the numbers at the end of the lines represent amino acid number in each species after aligning the sequences.

from the nucleotide sequence of the *glgC* gene. The structural gene consists of 1293 bp and codes for a protein of 431 amino acids (Baecker et al., 1983).

The finding of significant sequence similarity to the *E. coli* ADP-glucose pyrophosphorylase coupled with previous data showing altered kinetic parameters in various *sh2* mutants (Hannah and Nelson, 1976) are taken as confirming evidence that *Sh2* is indeed a structural gene for the maize endosperm ADP-glucose pyrophosphorylase.

A cDNA for the rice ADP-glucose pyrophosphorylase has been reported (Morrell et al., 1987b). Table 1 shows that comparison of the DNA sequence of this clone with that of *Sh2* detected little similarity; however, a comparison of the deduced amino acid sequence revealed 47% identity within the region of overlap. As predicted by the DNA sequence similarity, we detected no hybridization between the *Sh2* clone and the rice cDNA clone under normal hybridization and washing conditions (data not shown). However, the rice cDNA clone does hybridize to a maize endosperm cDNA clone. Subsequent investigations (J. Bae and L.C. Hannah, manuscript submitted) have shown that the identity of this clone is the *brittle-2* locus. This observation, coupled to earlier data (Hannah and Nelson, 1976) suggesting that *Bt2* encodes ADP-glucose pyrophosphorylase, leads us to conclude that the *brittle-2* locus is, in fact, a structural gene for this enzyme. The maize endosperm ADP-glucose pyrophosphorylase is thus composed of at least two dissimilar subunits; the subunits are the products of *Sh2* and *Bt2*.

A comparison of the *Sh2* deduced amino acid sequence with those of *E. coli*, a rice cDNA clone, and a recently published wheat endosperm cDNA (Olive et al., 1989) is given in Table 1 and Figure 5. Of particular significance is the finding that whereas the maize counterpart of the rice gene is *Bt2*, the derived amino acid sequences suggest that the rice gene and *Sh2* may also share a common origin; the rice gene, however, is more closely related to *Bt2*. The sequence comparison would also suggest that the recently described wheat gene is the more closely related counterpart of *Sh2*.

Overall, each of the plant polypeptides had 23% to 27% sequence similarity with the *E. coli* enzyme, whereas there was much greater similarity (37%) among the three plant proteins. Maize and wheat exhibited the greatest similarity (62%) with most of the sequence divergence appearing in the 5′ end of the cDNAs. Although not all clones were full length, the available data in Figure 5 coupled with the amino acid sequence derived from the spinach chloroplast subunits (Morrell et al., 1987a, 1987b) allowed us to conclude that the N-terminal sequence of the *E. coli* enzyme appears to be unique.

It is interesting that lysine 129 of the *E. coli* protein, an amino acid thought to be involved in activator binding (Parsons and Preiss, 1978a, 1978b), was not present in the wheat or maize protein. Whether the complementary subunit of these plant enzymes also lacks this binding site is yet to be elucidated. However, the activator-binding site of the small subunit (51 kD) of the spinach leaf enzyme is at the carboxyl end of the protein (Morrell et al., 1987a, 1987b). Figure 6 illustrates that all plant proteins show much sequence similarity in this region. All contain lysine at position 562 (Figure 5), an amino acid thought to be essential in activator binding (Olive et al., 1989). To summarize, the carboxyl terminus would appear to be important in activator binding.

Because lysine at position 291 is protected by enzyme substrates from chemical modification, this amino acid is

Table 1. Identity[a] of the Derived Amino Acid Sequence of ADP-Glucose Pyrophosphorylase of Maize with the Other Published Sequences

Species Compared	Amino Acids in Overlapping Reading Frame	Shared Amino Acids in Overlapping Reading Frame	% Identity in Overlapping Reading Frame
Maize/wheat/rice/*E. coli*	360	57	15.8
Maize/wheat/rice	384	142	37.0
Maize/wheat	534	333	62.4
Maize/rice	384	171	44.5
Maize/*E. coli*	483	125	27.2

[a] The identity has been calculated based on the alignment in Figure 5 as the proportion of identical residues in the overlapping regions. Therefore, the estimates do not take into account the size of the gaps between the overlaps and should be considered as approximate values.

apparently involved in an active site of the enzyme (Lee and Preiss, 1986; Parsons and Preiss, 1978a, 1978b). This residue is also found in *Sh2*, the rice protein, and in two of the three wheat-deduced polypeptides, suggesting that this amino acid is important in substrate binding in plants as well. Similarily, tyrosine 206 of *E. coli*, which is thought to bind adenine rings of ATP and ADP-glucose, is replaced in plants by another aromatic, hydrophobic amino acid, phenylalanine.

To summarize, although it is clear that the *E. coli* enzyme differs from the characterized plant systems in terms of the number of different subunits and the region involved in binding of the allosteric activator, the enzymes do appear to be similar in substrate binding sites. It is of particular interest to note that although plant enzymes are composed of two different subunits, much amino acid identity exists between the two subunits, and the two different coding genes may have a common origin via gene duplication and divergence.

DISCUSSION

The data presented here and past observations clearly identify the classically defined, starch-defective locus *Sh2* as a structural gene for one of the subunits of the starch synthetic enzyme ADP-glucose pyrophosphorylase of the maize endosperm.

The finding that *Sh2* encodes one of the subunits of this enzyme makes our earlier observation puzzling. Previously we showed that all of the analyzed *sh2* and all *bt2* mutants greatly reduced or abolished the activity of the major heat-labile form of the enzyme but only moderately reduced a minor heat-stable isoenzyme. It is possible that the collection of mutants analyzed previously represented a biased sample of all possible changes that could occur at the *Sh2* locus. Mutational alterations leading to approximately 10% wild-type levels are known to be almost wild type in phenotype, whereas those with less than 2% of the wild-type activity appear to be lethal (Hannah et al., 1980). Thus, the *sh2* mutants that are both phenotypically mutant and viable may represent a selected class of changes at this gene. Experiments to identify *Sh2* transcript and protein in these viable *sh2* mutants and to generate other *sh2* mutants are in progress.

As an alternative explanation, we have considered that there exists a minor endosperm ADP-glucose pyrophosphorylase that is independent of these two genes. Previous attempts to detect such an activity have yielded negative results. If such an activity exists, its purification properties must be identical to those of the major activity, and its kinetic parameters must, in some way, be altered by mutation at *Sh2* or *Bt2*. Although we find this possibility unlikely, we cannot definitively rule it out at this time. In fact, the finding that *sh2 bt2* double mutants contain detectable levels of enzyme suggests that such an activity may exist.

The genes encoding plant ADP-glucose pyrophosphorylases provide an interesting system for the study of two evolutionarily related but functionally separate genes. In the case of the maize endosperm, we (J. Bae and L.C. Hannah, manuscript submitted) have shown that the *Bt2*

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Spinach  1  S G I V T V I -(K)D A L I P S  15
Maize    1  S G I V - V I L K N A T I N -  15
Wheat    1  S G I V - V I Q K N A T I K -  15
Rice     1  S G I V T V I - K D A L L L A  15
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Figure 6. *Comparison of Putative Activator-Binding Sequences of Plant ADP-Glucose Pyrophosphorylase Polypeptides.*

The amino acid sequences of the putative activator-binding sites of polypeptides of maize (present work), wheat (Olive et al., 1989), and rice (Morrell et al., 1987a) were compared with the sequence determined for the activator-binding site of spinach enzyme (Morrell et al., 1987a, 1987b). Residue 9 in spinach was only tentatively identified as lysine (Morrell et al., 1988).

gene of maize is the counterpart of the rice ADP-glucose pyrophosphorylase structural gene discussed above. Because the two subunits of the plant ADP-glucose pyrophosphorylases share similarity at the polypeptide level, it seems reasonable that the two structural genes arose from a common progenitor. One might have expected that early in the evolutionary process the product of the duplicated locus and that of the original locus would be interchangeable. In fact, no data exist to rule out this possibility with almost all present-day plants. Because mutants exist in maize for both *Sh2* and *Bt2*, dominance and complementation patterns exhibited at these loci are relevant to this point. If the product of *Sh2* could substitute for that of *Bt2* locus, loss of enzymic activity would be seen only in an *sh2 bt2* double mutant. We observe, however, that mutation at *Sh2 or* at *Bt2* abolishes almost all enzymic activity and that mutants of *sh2* complement *bt2* mutants. Thus, in the case of the maize endosperm, the genetic data allow us to conclude that sequence divergence between the two loci has occurred to the point where they no longer function as duplicate loci but rather as complementary genes.

METHODS

Biological Material

The *Sh2* progenitor, the *sh2-m1* allele and its revertants were obtained in isogenic lines as described previously (Hannah et al., 1980; Tuschall and Hannah, 1982). The allele *sh2-m1* (mutable) has the controlling element *Ds* at the *sh2* locus, and the endosperms analyzed here lacked *Activator* (*Ac*). The *Sh2* revertants were obtained from a stock containing *Ac sh2-m1* and *a-m3*.

Isolation of DNA

High molecular weight DNA was purified from leaf material of the progenitor *Sh2* allele, *sh2-m1*, and phenotypically wild-type revertants Rev1 to Rev5 according to the procedure of Dellaporta et al. (1983). Restriction digestion, DNA gel blotting, and autoradiography were carried out as described in Maniatis et al. (1982). A 500-bp HindIII-PstI fragment of pES6-66 (Barton et al., 1986) was used as a probe for hybridization with the blots. Blot hybridizations and washes were carried out as described by Church and Gilbert (1984).

Isolation and Characterization of RNA

Total cellular RNA was extracted from kernels of the *Sh2* progenitor, the *sh2-m1* allele, and its revertants 22 days after pollination by the procedure of McCarty (1986). Isolation of the poly A^+ fraction of the total cellular RNA and RNA gel blot analysis to determine the size of the *Sh2* transcript were carried out as in Maniatis et al. (1982), using the same probe and hybridization protocol as used for DNA gel blots.

Construction and Screening of the cDNA Library

Complementary DNA from the poly A^+ fraction of mRNA from developing kernels of *shrunken-1*, *bronze-mutable4* (*sh1 bz-m4*) was constructed, EcoRI linkers were added, and cloning into λgt10 was done according to the protocols supplied by Bethesda Research Laboratories, Promega Corporation, and Davis et al. (1986) (D. St. Clair and L.C. Hannah, unpublished data). This library was screened for *Sh2* cDNA clones using the above-mentioned 500-bp probe and the same hybridization protocol as used for DNA gel blots.

Subcloning into Plasmids

Of 14 clones selected above, eight cDNA clones contained 1.95-kb inserts that had an internal EcoRI site separating them into a 900-bp and a 1050-bp EcoRI fragment each. The 900-bp and 1050-bp EcoRI fragments from one of these cDNA clones were subcloned into the EcoRI site of pUC119 (pc*Sh2*-1a and pc*Sh2*-1b, respectively). The 1.3-kb clone pES6-66 was also subcloned at the appropriate restriction sites of pUC119 as a 800-bp PstI-HindIII, a 500-bp HindIII-PstI, and a 250-bp PstI-EcoRI fragment (see Figure 3). The inserts were purified from the plasmids by restriction with appropriate enzymes and gel electrophoresis, followed by electroelution of the bands of interest (Maniatis et al., 1982) and used either as probes for various blots or for subcloning into phage M13 vectors and sequencing.

Subcloning into M13 Phage and Sequencing

The purified EcoRI inserts of pc*Sh2*-1a and pc*Sh2*-1b were subcloned into the EcoRI site of M13mp19 RF DNA. The 800-bp PstI-HindIII fragment of pES6-66 was subcloned into the PstI-HindIII sites of M13mp18 and M13mp19 RF DNAs. Identification and isolation of recombinant plaques, purification of single-stranded templates, and sequencing by the dideoxy chain-termination method (Sanger et al., 1977) were carried out as in the protocol supplied by Bethesda Research Laboratories. All three fragments were sequenced in both directions using the 17-base universal primers supplied by Bethesda Research Laboratories and New England Biolabs and synthetic 15-base primers made at the Interdisciplinary Center for Biotechnology Research-DNA Synthesis Core at the University of Florida. The sequences were analyzed and translated into amino acid sequence, and restriction maps were obtained using IBI sequencing programs. The restriction sites were also confirmed by digestion of the isolated inserts with appropriate enzymes whenever possible.

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