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Expression of ADP-Glucose Pyrophosphorylase in Maize (*Zea mays* L.) Grain and Source Leaf during Grain Filling

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The time course of ADP-glucose pyrophosphorylase activity and of starch accumulation rate measured in grain, from pollination to maturity, in *Zea mays* L. plants grown outdoors, was coincident for 2 years. No such correlation was observed in the adjacent leaf, which, furthermore, presented large year-to-year differences in starch accumulation pattern. Analysis of the expression of ADP-glucose synthase at the protein level, using antibodies directed against the Bt2 or Sh2 subunits, established that the variation of activity in the grain was explained by parallel changes in the content of both subunits. The cDNA for Bt2 and Sh2 subunits were used as probes to quantify the corresponding messenger. In grain, the time course of Bt2 and Sh2 mRNA accumulation anticipated, with a similar pattern, the specific peptide variations, which suggests a transcriptional control of expression. By contrast, the control of leaf activity by protein content was less obvious than in the grain, and changes in leaf enzyme specific activity were suggested during the first 20 d after pollination. A clone homologous to the grain Bt2 subunit cDNA was isolated from a maize leaf cDNA library, and a sequence comparison showed that the leaf clone (L2) was a partial cDNA representing one-third of the mature peptide. A 97% homology was observed between Bt2 and L2 in their coding region, but homology was poor in the 3′ noncoding border. This result demonstrates that Bt2 and L2 arise from different genes presenting a tissue-specific expression pattern and provides an explanation for the earlier reported differences between leaf and grain in the size of peptide and mRNA for the Bt2-homologous subunit.

Starch synthesis is dependent on ADP-Glc provided by ADPG-PPase (EC 2.7.7.27), which is considered a regulatory point in starch metabolism. Evidence for an important role of this enzyme in starch synthesis was first provided by mutants in which the starch deficiency, either in maize grain (Tsai and Nelson, 1966) or *Arabidopsis* leaves (Lin et al., 1988), was associated with loss of the enzyme activity. Further support was obtained recently by using transgenic plants underexpressing or overexpressing the enzyme: introducing a chimeric gene containing a region that is antisense to the coding region of one of the subunits of ADP-Glc pyrophosphorylase resulted in an abolition of starch formation and of enzyme activity in potato tubers, whereas the effect was less pronounced in leaves (Müller-Röber et al., 1992). Conversely, Stark et al. (1992) transformed tobacco, tomato, and potato with a bacterial mutant ADPG-PPase that was much less sensitive to phosphate inhibition. Starch accumulated at a higher rate in tissues expressing the modified enzyme, showing that the plant enzyme is rate limiting.

The enzyme is present in maize (*Zea mays* L.) grains and leaves, but starch synthesis is regulated differently in the two organs: starch is continuously accumulated in the endosperm during the filling period, whereas it is subjected to light-dark accumulation-depletion cycles in leaves. The starch synthesis pathway is also different because the precursor hexose-Ps are produced in the chloroplast, whereas they have to be imported from the cytosol in amyloplasts.

In maize, several starch-deficient mutants in grain were shown to be impaired in ADP-Glc pyrophosphorylase activity. Two of them described by Tsai and Nelson (1966) and Hannah and Nelson (1975) were shown to be complementary, suggesting that different genes encode the two subunits of the enzyme. Differential screening of a cDNA library from developing kernels with cDNA from the *shrunken-2* (*sh2*) mutant or wild type led recently to isolation of cDNA for the Sh2 subunit from endosperm (Bhave et al., 1990). The corresponding *brittle-2* (*Bt2*) subunit was cloned by homology to a rice endosperm clone (Bae et al., 1990). In parallel, it was shown that the larger subunit of the enzyme was missing in the endosperm of the *sh2* mutant and the smaller in the *Bt2* mutant (Preiss et al., 1990). However, the leaf enzyme did not seem to be affected by the *Bt2* and *Sh2* mutations, and, conversely, antibodies raised against the spinach leaf enzyme gave a lower response when probing endosperm proteins (Plaxton and Preiss, 1987; Preiss et al., 1990), which tends to indicate that different ADPG-PPase genes are encountered in leaf and grain expression. This hypothesis could also be supported by the slightly smaller size of the two subunits in the leaf compared to their equivalent in the endosperm

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Abbreviations: ADPG-PPase, ADP-Glc synthase/pyrophosphorylase (EC 2.7.7.27); DAP, days after pollination.

(Plaxton and Preiss, 1987; Spilatro and Preiss, 1987; Preiss et al., 1990). Krishnan et al. (1986), researching rice and wheat, and Villand et al. (1992), investigating barley, further observed tissue-specific differences in the size of the *Bt2*-type mRNA, which was larger in leaf (2.1 kb) than in endosperm (1.9 kb).

To better characterize the difference in the two ADPG-PPase forms we have compared the time course of enzyme activity, of quantity in both enzyme subunits, and of quantity of their RNA in grain and in the adjacent ear leaf during grain filling. For this purpose, a leaf-specific cDNA for one of the ADPG-PPase subunit was cloned and characterized.

MATERIALS AND METHODS

Plant Growth and Sampling

Maize (*Zea mays* L., cv F_7 F_2) plants were grown in a small experimental field at Orsay in 1988 and 1990 (as described by Prioul et al., 1990). Every 10 DAP five plants were taken at random. The leaf adjacent to the ear was sampled by punching discs (0.5 cm^2) and by cutting off a 12-cm^2 portion. At the same time, 25 grains were excised from the ear. All of the samples were immediately frozen in liquid nitrogen and stored at −70°C.

ADP-Glc Pyrophosphorylase

Enzyme activity was measured spectrophotometrically in the direction of ADP-Glc degradation in the presence of added PPi (Prioul et al., 1990). ADPG-PPase content was evaluated by an immunodot technique using antibodies raised against the Bt2 or Sh2 subunits from maize endosperm. The subunits were obtained at the University of Florida by purifying the peptides overexpressed in *Escherichia coli* after transformation with a plasmid (PET vector) bearing a 1.7-kb (Bt2) or a 1-kb (Sh2) portion of cDNA coding for the Bt2 and Sh2 subunits, respectively (Bae et al., 1990; Bhave et al., 1990). The antibody specificity and the ADPG-PPase subunit size were checked by western blotting. For this purpose, crude leaf extracts (10 μg of protein) were denatured and loaded on a 10% polyacrylamide gel, 0.1% SDS. After electrophoresis (4 h, 100 V, room temperature), the proteins were electrotransferred to a nitrocellulose membrane (Hybond C Extra, Amersham), and ADPG-PPase peptides were revealed as described below. An immunodot technique was used for quantification; it was carried out by spotting serially diluted crude extracts on the nitrocellulose (BA 85), using a Schleicher & Schuell apparatus. The membrane was immersed in 50% methanol, 2% H_2O_2 for 20 min, to destroy endogenous peroxidases, and then washed with water. Saturation of nonspecific sites was done by membrane immersion in 20 mM Tris-HCl (pH 7.5), 0.9% NaCl, and 0.5% Tween 20 with 50 g L^{-1} of defatted milk powder and 0.02% sodium azide for 45 min at 37°C. Incubation in primary antibody was done in the same buffer for one night at 4°C. The membrane was then incubated for 30 min in the secondary biotinylated antibody (goat anti-rabbit) and for 30 min in the streptavidine-peroxidase complex (ABC kit, Vector Laboratory). At each antibody change, three washings in 20 mM Tris-HCl (pH 7.5), 0.9% NaCl, and 0.5% Tween 20 buffer were done. Peroxidase activity was eventually developed in 100 mM Tris-HCl (pH 7.5), 0.8 mg mL^{-1} of diaminobenzidine, 0.4 mg mL^{-1} of $NiCl_2$, and 3 μL mL^{-1} of H_2O_2 at 3%. Photographs of the membrane were analyzed photometrically, to quantify the variations, as described by Prioul and Reyss (1988).

cDNA Library and Screening

Total RNA was extracted from leaves sampled 30 to 40 DAP by a guanidinium thiocyanate method and ultracentrifugation on $CsCl_2$ pads (Maniatis et al., 1982). At that time the leaf carbohydrate metabolism was very active for grain filling, and ADPG-PPase activity was reasonably high. Poly(A^+) RNA separated on oligo(dT)-cellulose column (Maniatis et al., 1982) was used for cDNA synthesis and ligation in λgt11 as described in the instruction manuals for the Pharmacia and Amersham kits. The leaf library was screened with a 650-bp cDNA (pAD_2) encoding a wheat endosperm ADPG-PPase (kindly provided by C.C. Ainsworth, Wye College, London University). Subsequently, its sequence was proved to be of the Bt2 type (Ainsworth et al., 1993). About 15 positive clones were screened from 50,000 to 100,000 phages plated out of the leaf library. One 0.7-kb clone, which presented a strong hybridization signal with the probe, was further analyzed. This clone, named L2, was subcloned in M13 phage at the *Eco*RI site, and its sequence was determined following the dideoxy termination method using a Pharmacia T7 polymerase sequencing kit. The L2 sequence (Fig. 8) showed a very high homology with the coding region of the *Bt2* cDNA from grain but a rather poor homology in the 3′ noncoding region. An attempt to further screen the same leaf library with the *Sh2* cDNA from maize endosperm for its leaf counterpart was unsuccessful, probably because of the low homology between the transcripts in the two organs as shown by the weak hybridization signal between the *Sh2* cDNA and leaf mRNA (see below).

RNA Quantification

Total leaf RNA was extracted as described by Logemann et al. (1987). The method was modified for grain RNA because the guanidine hydrochloride buffer solubilized starch from the endosperm, giving a viscous slurry, thus preventing any RNA extraction. One grain was ground in 1 mL of 10 mM Tris-HCl (pH 7.4), 1% SDS, 5 mM Na_2EDTA, 50 mM mercaptoethanol in a Potter homogenizer, and starch was immediately pelleted by a 5-min centrifugation at 4000*g* at 4°C. The supernatant was mixed with an equal volume of phenol:chloroform (1:1, v/v) and then treated as the leaf extract. Total RNA was quantified spectrophotometrically at 260 nm, and mRNA size for ADPG-PPase was checked by northern blotting. For this purpose, formaldehyde-agarose gels, sample preparation, and transfer on nitrocellulose membrane (Hybond N^+, Amersham) was performed as described by Fourney et al. (1988). Relative changes in ADPG-PPase-specific RNA were then evaluated by a hybridot technique: 5 μg of total RNA was serially diluted and spotted on nitrocellulose using a 96-well Schleicher & Schuell apparatus. Nucleic acids were fixed by a 5-min exposure under a UV lamp. Membranes were prehybridized and hybridized at

42°C in 50% formamide, 5× SSPE (0.18 M NaCl, 10 mM Na-P[pH 7.4], 1 mM EDTA), 0.5% SDS, 100 μg mL^{-1} of herring sperm DNA, 0.1% Ficoll, 0.1% PVP, 0.1% BSA. The probes were labeled by random priming with [P^{32}]dCTP and the Klenow fragment of DNA polymerase I (Maniatis et al., 1982). Membranes were washed with 2× SSPE, 0.1% SDS, twice for 5 min at room temperature and twice for 15 min at 65°C. An additional washing with 0.1× SSPE, 0.1% SDS at 60°C, twice for 10 min, was performed for homologous probes.

Genomic DNA and Southern Blots

DNA was extracted from leaves desiccated at 50°C for 16 h. Powdered material (5 g) was mixed with 9 mL of extraction buffer (100 mM Tris-HCl, 50 mM Na_2EDTA, 500 mM NaCl, 0.5% SDS) preheated at 65°C. After the mixture was incubated at 65°C for 15 to 30 min with agitation, 6 mL of saturated NaCl (>6 M) was added, and the mixture was shaken vigorously for 15 s. The tube was centrifuged for 20 min at 4000*g*, the supernatant was filtered on Miracloth, and 2 volumes of cold ethanol were added for nucleic acid precipitation. After the mixture was precipitated for 2 min at 4000*g*, the supernatant was discarded, the pellet was resuspended in 2 mL of buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, 0.5 M NaCl, containing 1 mg mL^{-1} of RNase A), and incubated for 1 h at 37°C. The insoluble debris was eliminated by a 15-min centrifugation at 4000*g*, and DNA was precipitated from the supernatant by 5 mL of cold ethanol. The floating DNA pellet, picked up with a plastic tip, was transferred to 10 mL of washing buffer (75% ethanol, 0.2 M Na_2-acetate) for 20 min and centrifuged for 2 min at 4000*g*. The pellet was further washed in 2 mL of 75% ethanol and 10 mM NH_4-acetate and finally resuspended in 0.5 mL in buffer (10 mM Tris-HCl, 1 mM Na_2EDTA).

DNA samples were electrophoresed in 0.7% agarose, dissolved in buffer (90 mM Tris-P, 2 mM EDTA) for 16 h (30 V/10 cm), as described by Maniatis et al. (1982). The DNA fragments (after depurination in 0.25 M HCl) were transferred onto a charged membrane (Hybond N$^+$, Amersham) by capillarity with 0.4 M NaOH. The membrane was prehybridized for 30 min and hybridized overnight in 0.5 M Na-P (pH 7.2), 7% SDS, and 1 mM Na_2EDTA at 67°C. The probes were labeled by random priming as described above. The membrane was washed two to three times in 40 mM Na-P (pH 7.2), 1% SDS at 65°C and exposed to Kodak XAR film at −80°C.

RESULTS

Starch Synthesis and ADPG-PPase Activity

Starch accumulation in the grain presented a characteristic pattern: a lag phase during the first 10 d (Fig. 1A), a linear increase from 10 to 16 and 35 to 40 DAP, and a progressive leveling off (Fig. 1). Grain ADPG-PPase activity was very low at the pollination stage, but it greatly increased at the time of maximum starch synthesis before declining by the completion of grain filling (Fig. 1). This pattern was rather constant from year to year (Fig. 1) regardless of the genotype (Prioul et al., 1990). Separate analysis of ADPG-PPase activity in the embryo showed a different time course with a maximum activity at 32 DAP and a fluctuation around a constant value thereafter (Fig. 2A). At any time, ADPG-PPase activity on a fresh weight basis was much lower in the embryo than in the whole grain (Figs. 1 and 2A). The contribution of embryo activity in total grain activity was negligible (<10%) except at the end (60 and 70 DAP) (Fig. 2B).

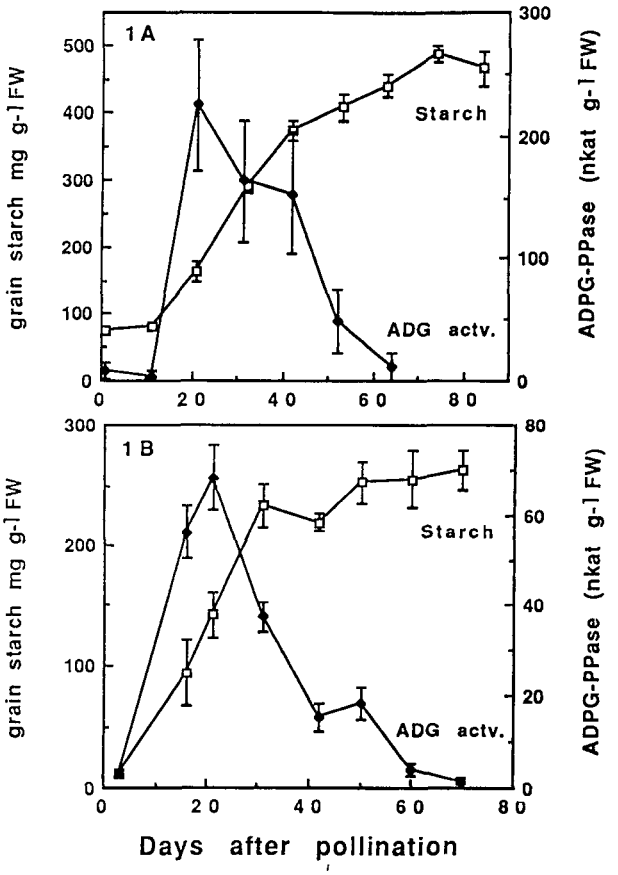

Figure 1. Time course of starch accumulation and of ADP-Glc pyrophosphorylase activity (actv.) in maize grain during filling. A, 1988; B, 1990. Results are means ± SE for samples from five plants. FW, Fresh weight.

A different situation was observed in the leaf adjacent to the ear. Large year-to-year differences were observed in the starch and ADPG-PPase time courses (Fig. 3). Activities and quantities were expressed on leaf area basis, which provided a better normalization of data than fresh or dry weight. However, fresh or dry weight/leaf area, a possible indicator of leaf thickness, did not vary greatly during the first 70 d. Afterward, a sudden decrease occurred that corresponded to the last step of leaf senescence leading to death (Prioul et al., 1990). The large error bars during that period reflected the large plant-to-plant difference in reaching that stage. ADPG-PPase activity peaked at 10 and 60 DAP in 1988 (Fig. 3A) or at 20 DAP in 1990 (Fig. 3B), but in both years the higher starch contents were associated with lower enzyme activities in four of five instances. Leaf starch is mainly dependent on the previous days' light condition and on sink demand. In these field experiments, the observed year-to-year differences

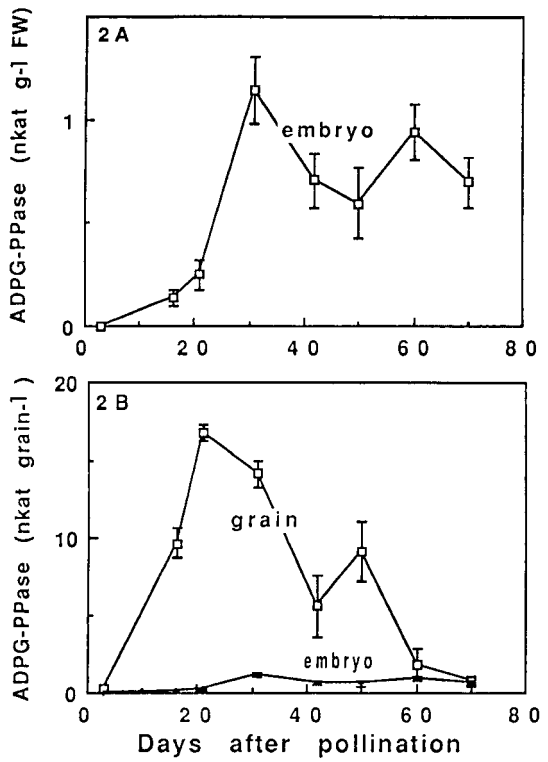

Figure 2. Time course of ADP-Glc pyrophosphorylase activity in maize embryo on a fresh weight basis (A) and comparison of activity per grain between total grain and embryo (B). Results are means ± SE for samples from five plants (1990). FW, Fresh weight.

are likely to reflect the unequal climate conditions and the correlative changes in source-sink balance.

ADPG-PPase Quantity and Specific mRNA Level

The variation in the quantity of enzyme relative to total protein was evaluated using antibodies raised against the Bt2 or Sh2 subunits of the enzyme, in the samples from 1990 experiment. In grain both antibodies gave a significant signal, whereas in leaf extracts only Bt2 antibodies could be used for quantification, because Sh2 antibodies gave a poor response (Fig. 4). Immunoblots from crude protein extracts separated by denaturing polyacrylamide gel electrophoresis confirmed that Sh2 subunits were larger than Bt2 subunits and that both were smaller in leaf than in grain (Fig. 4A). Sizes for all subunits were consistently smaller than reported earlier (Plaxton and Preiss, 1987; Spilatro and Preiss, 1987; Preiss et al., 1990). Independent measurement in our two laboratories yielded approximately 51 and 47 kD for Bt2 in grain and leaf, respectively, and 54 kD for Sh2 in grain. The size for Sh2 in leaf is uncertain due to poor recognition of the endosperm subunit antibodies. The new figure for Sh2 in maize grain is compatible with the size of 57 kD calculated from the genomic sequence, which includes a transit peptide of unknown length (Shaw and Hannah, 1992).

The relative quantity of RNA specific to each ADPG-PPase subunit was measured by separate hybridization of the same amount of total RNA to *Bt2*, *Sh2*, and L2 cDNA probes. Preliminary hybridization of total RNA separated on formaldehyde-agarose gels and transferred on nitrocellulose (northern blots, Fig. 5) showed that *Bt2* and *Sh2* probes gave similar signals with grain RNA at 2.0 and 2.2 kb, respectively. Probes to *Bt2* and L2 both hybridized to the same size band either in grain or in leaf extracts, but the mRNA had a smaller size in grain than in leaf: 2.0 versus 2.25 kb. Band labeling for leaf RNA was relatively stronger with L2 than with *Bt2*, suggesting a higher homology of the L2 probe (Fig. 5). Hybridization of the *Sh2* cDNA with leaf RNA was extremely weak (not shown), suggesting that this grain probe has a poor homology with the RNA coding for the leaf equivalent of the Sh2 subunit. This result probably explains our failure to isolate a leaf *Sh2* clone from our library. Therefore, the hybridot technique used for RNA quantification could only be applied to transcripts of Bt2 and Sh2 subunits in grain and of L2 in leaf.

In grain the relative quantity of both subunit peptides varied in parallel (Fig. 6A) as expected from the reported equimolar ratio of the subunits in the holoenzyme (Plaxton and Preiss, 1987). The variation of subunit quantity was very

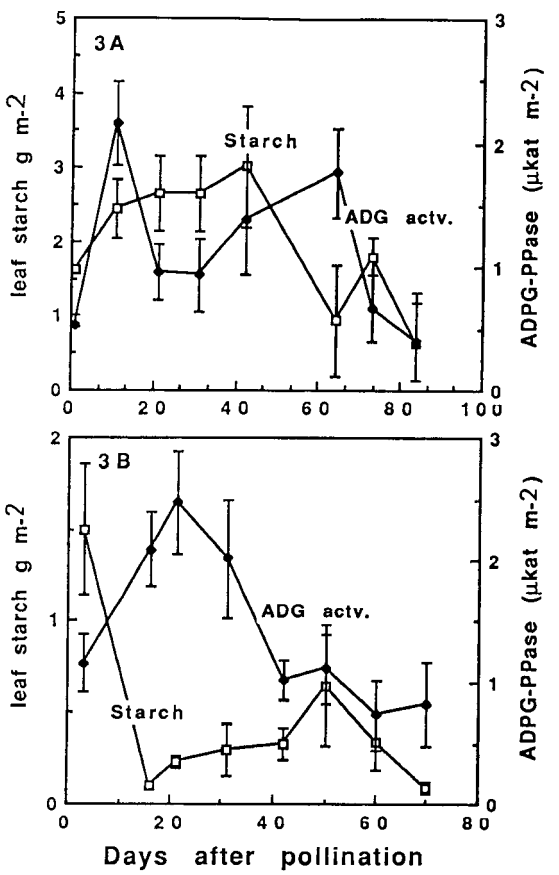

Figure 3. Time course of starch content and of ADP-Glc pyrophosphorylase activity (actv.) in the leaf adjacent to the ear during grain filling. A, 1988; B, 1990. Leaves were sampled at the end of the morning, i.e. after a 5- to 8-h photosynthesis period. Results are means ± SE for samples from five plants.

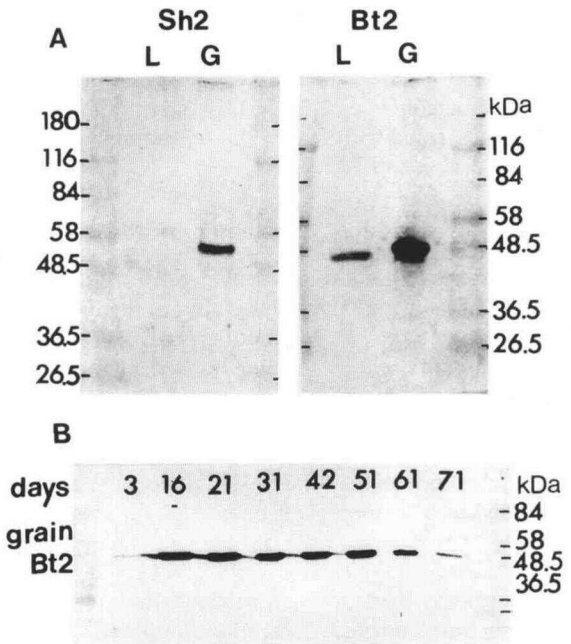

Figure 4. Western blot analysis of maize leaf and grain ADP-Glc pyrophosphorylase. A, Compared size of the two subunits in leaf and grain extracts. Total soluble protein was separated on a 10% polyacrylamide gel in denaturing conditions (0.1% SDS), transferred on nitrocellulose, and probed with antibodies raised against the Sh2 (left) or the Bt2 (right) subunit of the enzyme. L, Leaf extract; G, grain extract. B, Time course of relative quantity in the grain Bt2 subunit after pollination. Each lane was loaded with 10 μg of protein extract sampled 3 to 71 DAP (1990 experiment).

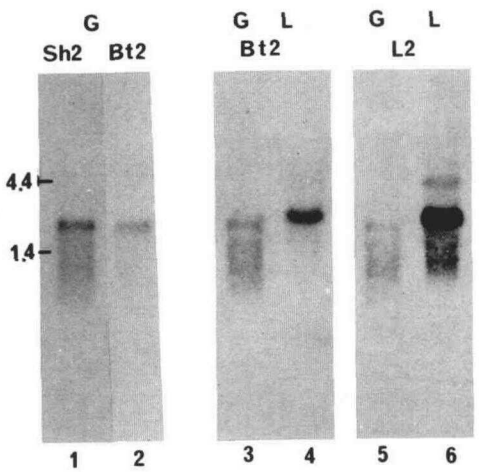

Figure 5. Northern blot analysis of grain and leaf RNA. Total RNA (10 μg) was separated on a formaldehyde-agarose gel, transferred on nitrocellulose, and hybridized to the *Bt2* and *Sh2* cDNA clones from endosperm ADP-Glc pyrophosphorylase (Bae et al., 1990; Bhave et al., 1990) and to a cDNA clone from leaf (L2). Position of the size markers (kb) are indicated on the left. G, Grain extract; L, leaf extract. Lane 1 was hybridized with *Sh2* cDNA; lanes 2, 3, and 4 with *Bt2* cDNA; and lanes 5 and 6 with L2 cDNA. Samples are from 1990 experiment.

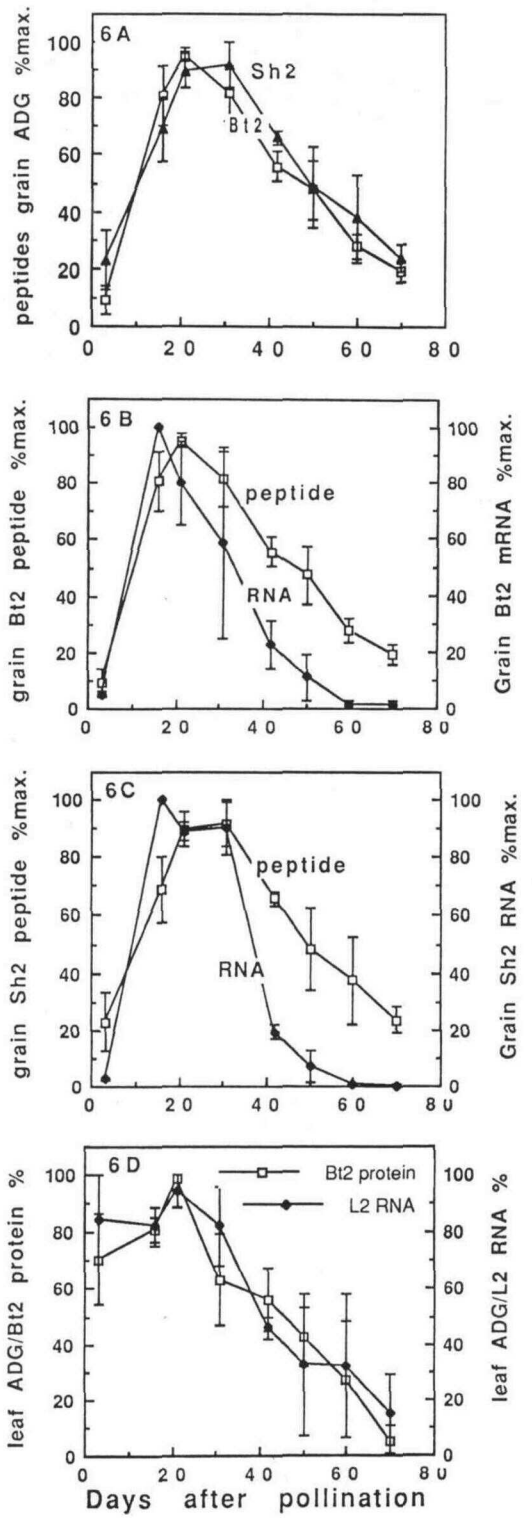

Figure 6. Time course of expression of ADP-Glc pyrophosphorylase subunits and their mRNA in grain and leaf during filling. Relative quantities were evaluated by the immunodot or hybridot techniques. A, Bt2 and Sh2 peptides in grain; B, grain Bt2 peptide compared to its mRNA; C, Sh2 peptide compared to its mRNA; D, leaf Bt2-homologous peptide compared to L2 mRNA. Samples are from 1990 experiment; results are means ± SE for samples from three plants.

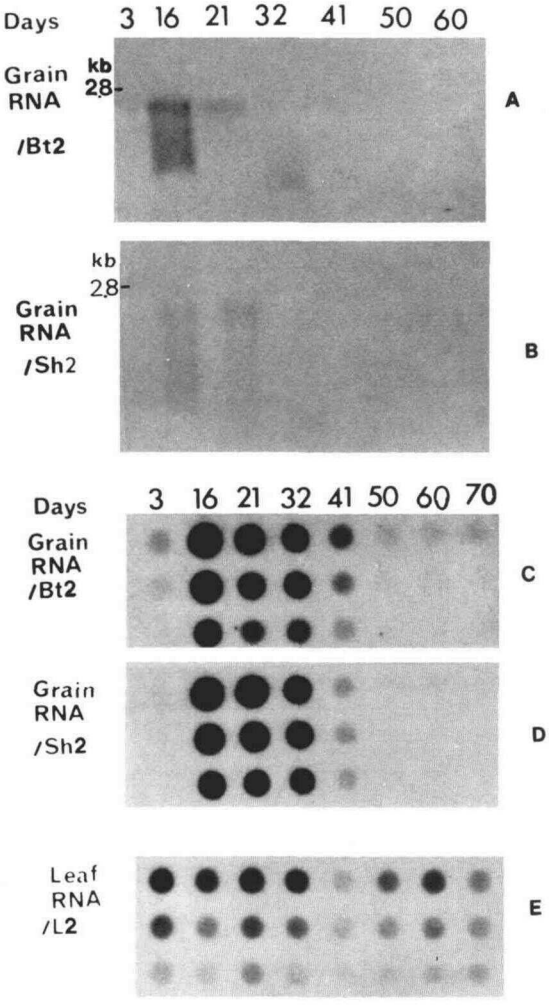

Figure 7. Northern blots and hybridots of grain or leaf RNA during grain filling. Hybridization of 10 μg of grain RNA, electrophoresed on agarose-formaldehyde gels, transferred on nitrocellulose with *Bt2* probe (A) and with *Sh2* probe (B). Hybridization of total RNA spotted on nitrocellulose (C, D, and E). Serial dilution from 5 μg (first row) to 1.25 μg (third row) of grain RNA hybridized with *Bt2* probe (C), with *Sh2* probe (D), and of leaf RNA hybridized with L2 probe (E). Samples from 1990 experiment.

similar to that of activity (Figs. 1B, 4B, and 6A), which confirms our preliminary observations with antibodies to the native enzyme (Prioul et al., 1991). However, the amount of both peptides was still 20% of maximum at 70 DAP when activity was close to zero (Fig. 1B). This suggests that the decline in enzyme activity in grain was not totally controlled by protein down-regulation. The relative quantity of *Bt2* and *Sh2* mRNA, based on same amount of total RNA, peaked at 16 DAP and decreased to a very low value after 50 DAP (Figs. 6, B and C, and 7, C and D). Examination of northern blots showed a strong tendency toward mRNA degradation in grain after 21 DAP (Fig. 7, A and B). In both cases, the variation in mRNA preceded the one in the corresponding peptide (Fig. 6, B and C).

In leaves enzyme quantity remained relatively stable during the first 21 DAP (Fig. 6D), whereas ADPG-PPase activity nearly doubled (Fig. 3B). The decline in both enzyme content and activity was then rather similar (Figs. 3B, 6D, and 7E). The same time course of the mRNA content probed with the L2 cDNA coincided with that of the Bt2-homologous protein (Fig. 6D).

Sequence Comparison of L2 with *Bt2* and *Sh2* cDNA

The sequence of the L2 clone screened from our leaf cDNA library (Fig. 8) showed an open reading frame of 125 amino acids terminated by a stop codon and 236 nucleotides corresponding to the 3′ noncoding border. Alignment of this sequence to the *Bt2* cDNA from grain (Bae et al., 1990) indicated a very high homology (15 of 380 nucleotide changes) between L2 and the *Bt2* 3′ coding region. However, a large divergence appeared in the 3′ noncoding border (Fig. 8). The deduced amino acid sequence of L2 presented four of 125 changes and one extra amino acid residue compared to Bt2. In contrast to this, L2 and *Sh2* DNA sequences shared a low homology, but some conserved regions appeared when comparing the deduced L2 and Sh2 amino acid sequences (Fig. 9). As a whole, the sequence comparisons proved that L2 is a partial sequence of the leaf counterpart of the Bt2 subunit, but the large differences in the noncoding region are consistent with the involvement of two different genes. This

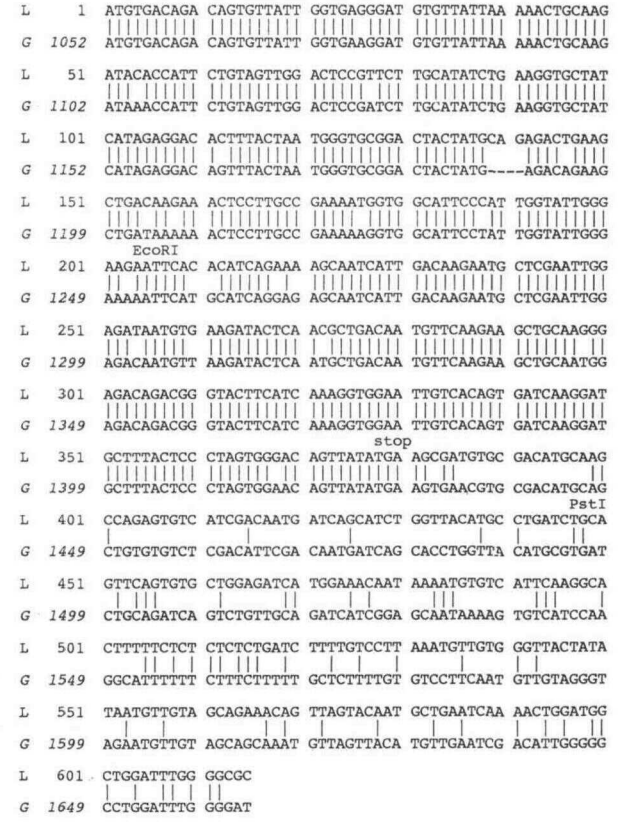

Figure 8. Homology between sequences of the L2 clone from leaf cDNA and of the *Bt2* sequence from maize endosperm (Bae et al., 1990).

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leaf.   1 VTDSVIGEGC VIKNCKIHHS VVGLRSCISE GAIIEDTLLM GADYYAETEA
          |||||||||| ||||||| || |||||||||| |||||| ||| ||||| ||||
bt2   352 VTDSVIGEGC VIKNCKINHS VVGLRSCISE GAIIEDSLLM GADYY-ETEA

leaf .51 DKKLLAENGG IPIGIGKNSH IRKAIIDKNA RIGDNVKILN ADNVQEAARE
         ||||||| || |||||||||  || ||||||| |||||||||| |||||||| |
bt2   395 DKKLLAEKGG IPIGIGKNSC IRRAIIDKNA RIGDNVKILN ADNVQEAAME

leaf.101 TDGYFIKGGI VTVIKDALLP SGTVI
         |||||||||| |||||||||| |||||
bt2   445 TDGYFIKGGI VTVIKDALLP SGTVI


leaf  1   VTDSVIGEGC VIKNCKIHHS VVGLRSCISE GAIIEDTLLM GADYYAETE-
              |  ||     | | || | |  |  |  |     |  | ||| | |||
sh2   418 KMKYAFISDGC LLRECNIEHS VIGVCSRVSS GCELKDSVMM GADIY-ETEE

leaf .50 -ADKKLLAENGGI PIGIGKNSH IRKAIIDKNA RIGDNVKILN ADNVQEAARE
          | ||||| |   ||||| |   ||  ||| || ||| || | |     |||
sh2   468 EASKLLLA--GKV PIGIGRNTK IRNCIIDMNA RIGKNVVITN SKGIQEADHP

leaf.101 TDGYFIKGGI VTVIKDALLP SGTVI
          || |  || |  | |      ||
sh2   518 EEGYYIRSGI VVILKNATIN ECLVI
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Figure 9. Homology between the deduced amino acid sequence from leaf L2 cDNA and those of the Bt2 and Sh2 peptides from endosperm ADPG-PPase (Bae et al., 1990; Bhave et al., 1990).

hypothesis was further tested by hybridization of L2 and *Bt2* probes to genomic DNA from the parent inbred lines (F_2 and F_7) of the hybrid (F_2F_7) used for cDNA cloning and one line (W22) for which a genomic library is commercially available (Clontech). Distinct patterns (Fig. 10) were observed with the three inbred lines and two restriction enzymes. Multiple bands were noted with *Bt2* and fewer bands with L2; therefore, a multigenic family is encountered. As expected from the partial homology between probes, at least one band was in common. However, bands specific to L2 also showed up, which indicates that L2 and *Bt2* are likely to be different genes rather than alleles of the same gene. Chromosomal location of both genes, using restriction fragment length polymorphism mapping with a recombinant inbred line, showed a common locus on chromosome 1 (M. Causse, D. de Vienne, J.P. Rocher, and J.L. Prioul, unpublished data). *Bt2* mutant was previously mapped on chromosome 4 (Teas and Teas, 1953). Genomic clones specific to *Bt2* and L2 were also isolated from the W22 library (not shown).

DISCUSSION

The grain filling period is when an important change in carbon allocation pattern takes place in maize plants. Most of the carbohydrates imported by grain come from assimilates photosynthetically fixed by source leaves after pollination (Cliquet et al., 1990; Prioul et al., 1990). Although metabolites and enzymes involved in carbohydrate metabolism are quite similar in both sources and sinks, they are regulated differently. For example, in leaves, starch represents a transient reserve accumulated during the light period, which can be used for Suc export during the night, whereas in the grain, after the initial 20 DAP lag phase, starch accumulates at a constant rate during a prolonged period regardless of the external conditions or the genotype (Prioul et al., 1990). A way to analyze the regulation of this metabolism is to identify the regulatory points and to try to understand their functioning. The analysis of mutants has been a great help. A number of maize genotypes, deficient in starch endosperm, have been isolated by their shrunken or brittle kernel phenotypes. They have been shown to be impaired in either Suc synthase or ADP-Glc pyrophosphorylase activities in the endosperm but not in the embryo (Hannah and Nelson, 1975; Chourey and Nelson, 1976). It was demonstrated that Suc synthase is encoded by two genes expressed either mainly in the endosperm (*Sh1*) or mainly in the vegetative organs (*Sus*) (Chourey and Latham, 1986), although there was no difference in specific activity of the two isoforms (Nguyen-Quoc et al., 1990).

The situation is even more complex for ADPG-PPase because the two subunit types of the maize endosperm enzyme are encoded by two different genes, corresponding to the *Bt2* and *Sh2* mutations (Tsai and Nelson, 1966; Hannah and Nelson, 1975). Because none of these mutations apparently affected the enzyme in embryo and leaf, other genes are probably involved. Several observations tend to indicate that enzyme expression in grains could be controlled by grain-soluble carbohydrates: aborting kernels showed a failure to transitorily accumulate soluble sugars and then to synthesize ADPG-PPase (Hanft and Jones, 1986; Prioul and Schwebel-Dugué, 1992). The Suc concentration at 20 DAP and ADPG-PPase activity were correlated in mutants partially impaired in starch accumulation (Doehlert and Kuo, 1990).

In the present study, we confirm the parallel variation of ADPG-PPase activity and starch accumulation rate in maize grain reported during the first 28 DAP by Tsai et al. (1970), Ozbun et al. (1973), and throughout the filling period by Prioul et al. (1990). We further show that this pattern may be largely explained by simultaneous changes in the quantity of both ADPG-PPase subunits (Fig. 6A). Variation in the relative content of *Bt2* and *Sh2* mRNA anticipated those of the corresponding peptide, which could suggest a transcriptional control of ADPG-PPase expression in grain (Fig. 6, B and C). Similar trends have been previously reported in

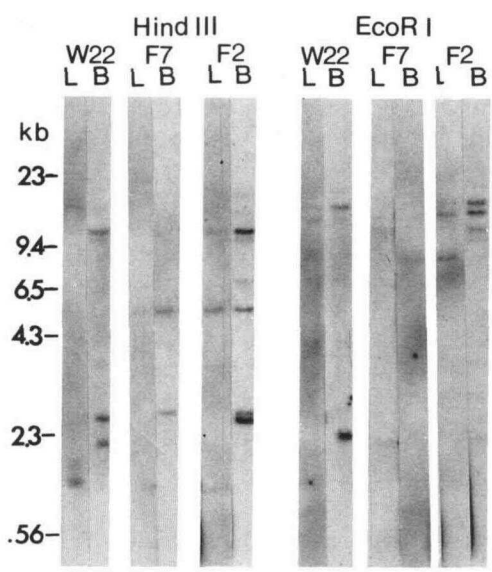

Figure 10. Southern blots of genomic DNA extracted from three inbred lines (W22, F_7, F_2), digested by two restriction enzymes, *Eco*RI and *Hin*dIII. The same membrane was hybridized sequentially with L2 (L) and *Bt2* (B) cDNA probes. Molecular size markers are on the left.

wheat grain with one of the ADPG-PPase subunits at both the mRNA and protein level and was also correlated with time course in starch synthesis rate (Reeves et al., 1986). The present correlative variation of both RNAs is at variance with results reported by Müller-Röber et al. (1990) in potato leaves, in which starch accumulation was linked to RNA level for the S subunit (homologous to maize Sh2) but not for the B subunit RNA (homologous to maize Bt2). However, the same authors reported that antisense RNA to subunit B strongly inhibited ADPG-PPase expression in transgenic potato plants (Müller-Röber et al., 1992).

Tissue-specific differences in ADPG-PPase expression become evident when comparing the grain with the adjacent leaf during grain filling. The time course for leaf activity was year dependent: In 1988, leaf carbohydrate content was low during the 20- to 50-DAP period corresponding to the maximum rate of grain starch synthesis, reflecting a high sink demand and a limiting supply, whereas in 1990, assimilates provided enough supply to avoid draining of leaf carbohydrate reserves. In both years, there was frequently an opposite relationship between leaf starch accumulation and ADPG-PPase activity. Such an observation is rather common in leaves either during a diurnal cycle (Jeannette and Prioul, 1992, in maize; Li et al., 1992, in sugarbeet) or when comparing genotypes (Rocher et al., 1989, in maize). This means that in addition to the P glycerate/Pi ratio some unknown factors may control ADP-Glc synthesis rate in vivo (for specific discussion, see Li et al., 1992).

Variations in protein amounts represent another level of regulation. The link between enzyme activity and ADPG-PPase Bt2-homologous protein content is less obvious in leaf than in grain, especially for the first 20 DAP when activity doubled in the leaf with no significant change in protein level (cf. Figs. 3B and 6B to 1B and 6A). This suggests a variation in leaf enzyme specific activity consistent with those inferred from diurnal variations (Jeannette and Prioul, 1992). Another large difference between grain and leaf is in the magnitude of the changes in activity: 1 to 100 in grain and 1 to 2 in leaf from 0 to 20 DAP. Relative content of the Bt2 protein content and of the corresponding mRNA also had an organ-specific time course. In both organs the mRNA variations could explain those in the protein (Fig. 6).

Sequence comparison demonstrated that the gene expressing the smaller subunit of ADPG-PPase (Bt2 type) in endosperm is actually different from that in leaf, due to the large differences in the 3′ noncoding region. This analysis supports the hypothesis proposed by Krishnan et al. (1986) from their observation of tissue-specific differences in peptide and mRNA sizes for ADPG-PPase subunits. We also confirm that *Bt2* mRNA was slightly larger in leaf than in grain (2.25 versus 2.0 kb), whereas the opposite was observed for the peptides (48 versus 51 kD). A similar discrepancy between RNA and peptide size for the ADPG-PPase small subunit in leaf and grain was mentioned in rice, wheat (Krishnan et al., 1986), and barley (Villand et al., 1992). The striking homology in the carboxy-terminal part of endosperm and leaf Bt2 peptide is consistent with a similar role in the holoenzyme. For example, a large part of the amino acid sequence carrying the activator 3-P glycerate-binding site of the spinach enzyme, GIVTVIKDAL (Morell et al., 1988) was totally conserved in maize *Bt2* genes, expressed in endosperm or leaf, and located just before the C-terminal end of the peptide (Fig. 9). The high homology in Bt2-type subunits between species and organs was confirmed when comparing L2 with pAD2 sequence from wheat grain and barley (C.C. Ainsworth, personal communication; Villand et al., 1992). A similar conclusion was raised by Smith-White and Preiss (1992) in an extensive comparison of 11 published protein sequences from monocotyledonous or dicotyledonous ADPG-PPase. They further suggested, from a comparison of wheat grain and leaf sequences published by Olive et al. (1989), that tissue specificity could mainly originate from differences in the N-terminal region, which was apparently more variable in length. Our observation of an excellent conservation in the 3′ coding region of *Bt2* and L2 cDNA supports this proposition in maize. Further refinement in tissue specificity for ADPG-PPase may occur in C_4 plants like maize because Spilatro and Preiss (1987) have described significant differences in the biochemical properties of ADPG-PPase from mesophyll cells and bundle sheath strands. It would be interesting to know whether different genes encode for these isoforms. The multiband pattern observed in Southern blots (Fig. 8) would be consistent with this hypothesis.

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