Plant Physiol. (1994) 106: 713-722

# Coordinated Transcriptional Regulation of Storage Product Genes in the Maize Endosperm[1]

**Michael J. Giroux, Charles Boyer, Gunter Feix, and L. Curtis Hannah***

Department of Horticultural Sciences and Program in Plant Molecular and Cellular Biology, University of Florida, Gainesville, Florida 32611 (M.J.G., L.C.H.); Department of Horticulture, Pennsylvania State University, University Park, Pennsylvania 16802 (C.B.); and Institut fur Biologie III, Universitat Freiburg, 79104 Freiburg, Germany (G.F.)

**We have demonstrated that expression of genes involved in starch and storage protein synthesis of the maize (*Zea mays* L.) endosperm are coordinated. Genetic lesions altering synthetic events in one biosynthetic pathway affect expression of genes in both pathways. Initial studies focused on *shrunken2* (*sh2*) and *brittle2* (*bt2*) mutants because these genes encode subunits of the same enzyme, ADP-glucose pyrophosphorylase. Analysis of various *sh2*- and *bt2*- mutant alleles showed that the most severe mutations also conditioned the largest increase in transcripts. The analysis was extended by monitoring the transcripts of the genes, *shrunken1* (*sh1*, structural gene for Suc synthase), *sh2, bt2, waxy1* (*wx1*, structural gene for starch synthase), and those of the large and small zeins in isogenic maize lines at 14, 22, and 30 d postpollination. Endosperms were wild type for all of these genes or contained *sh1-*, *sh2-*, *bt1-*, *bt2-*, *opaque2* (*o2-*), or *amylose-extender1* (*ae1-*) *dull1* (*du1-*) *wx1-* mutations. Transcripts increased continually throughout kernel development in the mutants relative to the standard W64A used. Variation in the amount of Suc entering the developing seed also altered transcript amounts. The results indicate that starch and protein biosynthetic genes act in a concerted manner, and both are sensitive to mutationally induced differences.**

The maize (*Zea mays* L.) endosperm is a specialized tissue that provides a source of nutrients to the germinated embryo. The vast majority of carbon used in the early steps of seedling development is derived from starch and the storage proteins, the zeins. These storage products account for approximately 90% of the dry weight of a mature maize endosperm.

Historically, mutants have provided the most insight into the pathways of starch and zein synthesis. Many endosperm mutations can be classified as affecting starch or zein biosynthesis. Mutations such as *o2-*, *floury2-*, and *o7-* decrease zein content, and proteins in the other solubility classes increase in abundance (Mertz et al., 1964; Nelson et al., 1965; Ma and Nelson, 1975; Habben et al., 1993). *o2* has been shown to encode a *trans*-acting regulatory protein involved in zein transcription (Schmidt et al., 1990). S and L zeins represent the 19- and 22-kD classes of zein polypeptides and an *o2*-mutation affects mainly the production of 22-kD zein proteins (Langridge et al., 1982).

The majority of the enzymes known to be involved in the starch biosynthetic pathway follow similar developmental timing (Tsai et al., 1970). Ingle et al. (1965) found maximum endosperm sugar content at 22 dpp. This stage of development is also when the starch biosynthetic enzymes are most active (Tsai et al., 1970). Mutations in the genes *sh1* (the structural gene for Suc synthase; Chourey and Nelson, 1976), *bt1* (the structural gene for a membrane-bound, adenylate carrier; Sullivan et al., 1991), and *sh2* and *bt2* (both structural genes for AGP; Hannah and Nelson, 1976) decrease starch content in the mature kernel as a consequence of the loss of an important starch synthetic enzyme or protein. Mutations in *wx1* (the structural gene for the starch-bound ADP-Glc glucosyl transferase; Nelson and Rines, 1962), *ae1* (the structural gene for a starch-branching enzyme; Boyer and Preiss, 1978), and *du1* (Creech, 1965) do not reduce starch amount but rather alter the ratio of the two major starch constituents, amylose and amylopectin. The straight-chained amylose is abolished in *wx1-* mutations, whereas *ae1-* and *du1-* mutations increase the amount of amylose relative to the amount of amylopectin. Neither *ae1-* nor *du1-* reduces the total amount of starch by more than 20% (Creech, 1965). The triple mutant *ae1- du1- wx1-* results in kernels with increased sugars and reduced starch similar to *sh2-*, *bt2-*, and *bt1-* mutants (Creech, 1965). More detailed descriptions of these mutants can be found in a recent review (Hannah et al., 1993).

Although mutations are historically placed into the starch or zein biosynthetic pathway, hints of possible interactions between these pathways have been noted. For example, Tsai et al. (1978) and Barbosa and Glover (1978) reported that the *sh2-*, *bt2-*, and *bt1-* mutations that severely reduce starch synthesis also reduce zein synthesis to the same extent as *o2-*.

Ample evidence exists that expression of various genes is modulated by sugar concentration. For example, studies with maize protoplasts revealed repression of transcription of photosynthetic genes by various carbon sources (Sheen, 1990).

[1] Contribution from the Florida Agricultural Experiment Station Journal Series, No. R-03868.

* Corresponding author; fax 1-904-392-6479.

Abbreviations: *ae1*, amylose-extender1; AGP, ADP-Glc pyrophosphorylase; *agp1*, ADP-Glc pyrophosphorylase1; *agp2*, ADP-Glc pyrophosphorylase2; *bt1*, brittle1; *bt2*, brittle2; dpp, days postpollination; *du1*, dull1; *L zein*, large zein; *o2*, opaque2; *S zein*, small zein; *sh1*, shrunken1; *sh2*, shrunken2; *sus1*, Suc synthase1; *su1*, sugary1; *wx1*, waxy1.

In potato, expression of one of two starch AGP genes was increased in response to elevated Suc concentrations (Muller-Rober et al., 1990). With regard specifically to maize starch biosynthetic genes, Koch et al. (1992) showed that expression of wild-type alleles of *sh1* and *sus1*, the genes encoding the two forms of Suc synthase, was modulated in maize roots by sugar content. The amount of Suc leading to maximal *sh1* expression was less than that for *sus1*. These observations, taken together, suggest that the response of these genes relates to their role in tissues serving as a source or sink for carbohydrates. Accordingly, the maize endosperm might be expected to respond to high sugar concentrations by increasing the amount of transcripts present. In previous studies (Bae et al., 1990), we noted that mutation at either of the two structural genes (*sh2* and *bt2*) for AGP resulted in increased steady-state levels of transcripts of the other nonallelic and nonmutant structural gene. Further studies were designed to focus on the coordination of expression between these two genes as detected by mutational analysis. It was discovered that this networking in expression is more global than just these two genes, reflects the severity of the genetic block, and involves the expression of genes important for at least starch and protein synthesis. The mutant effect can be mimicked in wild-type seed by increases in the sugars presented to developing kernels. The results of those studies are the subject of this report.

## MATERIALS AND METHODS

### Nomenclature

Names and symbols for genes, their alleles, and their products follow the 1993 version of maize genetics nomenclature (Beavis et al., 1993). When reference is made to the gene or to its alleles, the abbreviation is italicized; for example, *sh2* is used to refer to this locus. Alleles beginning with a capital letter, e.g. *Sh2-*, refer to the functional or dominant form of the allele, whereas recessive alleles are designated with a lowercase first letter, e.g. *sh2-*. A hyphen follows the allelic symbol and the designation of the particular allele, e.g. *sh2-R*, is given when known or relevant. Gene products are not italicized. Proteins are given in all capital letters, e.g. SH2. Transcripts, as suggested by Oliver Nelson, chairman of the Maize Nomenclature Committee, are distinguished by the nonitalicized gene symbol; the first letter is capitalized when the transcript arises from a functional wild-type allele.

### Plant Culture

The *sh2-* and *bt2-* mutant alleles were described previously (Hannah and Nelson, 1975, 1976). The W64A isogeneic lines were wild type or contained the mutant alleles, *sh1-*, *sh2-*, *bt1-*, *bt2-*, *o2-*, *ae1-*, *du1-*, or *wx1-* as well as *ae1- du1- wx1-*. The W64A isolines were grown in a greenhouse with temperatures of 33°C (day) and 24°C (night). Developing ears were utilized as a source of kernels for RNA, protein, and run-on transcription analysis.

### RNA Isolation

RNA was isolated from kernels that had been quick-frozen in liquid $N_2$. The samples were ground to a fine powder with a mortar and pestle, and RNA was isolated by the LiCl method of McCarty (1986). Total RNA was utilized in the northern blot analysis.

### RNA Gel Blots

RNA samples were denatured in 2.2 M formaldehyde/50% formamide and fractionated on 1.5% agarose/2.2 M formaldehyde gels using a Mops buffer (Maniatis et al., 1982). RNA gels were blotted onto nylon membrane (Hybond N, Amersham) following standard protocols (Maniatis et al., 1982). The transfer buffer was 10× SSPE (1.8 M NaCl, 100 mM $Na_2HPO_4$ [pH 7.7], and 10 mM EDTA). After blotting, samples were cross-linked to the wet nylon membrane by 6 min of UV irradiation (3 J/cm$^2$).

Hybridizations were in 0.5 M $Na_2HPO_4$ (pH 7.2), 7% SDS, and 1% BSA (Church and Gilbert, 1984). Blots were prehybridized for 1 h at 65 to 67°C, and hybridizations were for 16 to 20 h, also at 65 to 67°C. Probes were prepared by the random primer method (BRL protocols) to a specific activity of greater than $5 \times 10^8$ cpm/$\mu$g cDNA. Blots were washed twice in 2× SSPE, 0.1% SDS and twice with 0.1× SSPE, 0.1% SDS. Each wash was for approximately 30 min at 65 to 67°C. Filters were dried and subjected to autoradiography. Signals were quantified using an Ambis radioanalytical imaging system. Filters were reprobed with an rDNA clone, and data were adjusted for minor loading discrepancies. Data in Tables I, II, and III using replicated RNA or nuclei samples from kernels of greenhouse-grown maize gave values that were within ±25%.

### Protein Analysis

Tissue for protein samples was ground to a fine powder with a mortar and pestle in liquid $N_2$ and added to an equal weight:volume of buffer containing 0.1 M Tris-HCl (pH 7.8), 10 mM DTT, and 20% glycerol. 2-Mercaptoethanol was added to 5%, and the samples were heated to 90°C for 3 min. Protein determinations were as described by Bradford (1976) using a BSA standard. SDS-PAGE was by the method of Laemmli (1970). Proteins were transferred to nitrocellulose membranes (BA-85 0.45 $\mu$m, Schleicher & Schull) by standard protocols (Ausubel et al., 1987). Ponceau S staining provided visual confirmation of even protein loading and electroblot transfer (Ausubel et al., 1987).

Immunoblots were performed according to protocols supplied by Bio-Rad using 1% BSA to block nonspecific antibody binding. A 1:300 dilution of SH2, BT2, or preimmune antiserum in Tris-buffered saline with 1% BSA was used. The preparation of the SH2 and BT2 antibodies was described previously (Giroux and Hannah, 1994). Antibody binding was recognized by binding of the secondary antibody, goat anti-rabbit (Bio-Rad), linked to an alkaline phosphatase conjugate and developed with the color reaction utilizing 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (BRL; Ausubel et al., 1987).

SH2 and BT2 protein levels were quantified using enhanced chemiluminescence protocols and materials (Amersham). Development of the blots utilized the binding of the secondary antibody, goat anti-rabbit (Bio-Rad), linked to a

**Table I.** *Activity of AGP in endosperms and embryos of sh2- and bt2-mutants*

Dry weights were obtained from 22-dpp kernels. The AGP activities in the mutants are relative to the standard W64A × 182E $F_2$ endosperm and embryos.

| Genotype | Dry Wt | Relative AGP Activity | |
|---|---|---|---|
| | | Endosperm | Embryo |
| WT | 0.0968 | 1.00 | 1.00 |
| *sh2-I* | 0.0760 | 0.38 | 1.06 |
| *sh2-R* | 0.0696 | 0.19 | 1.44 |
| *bt2-B* | 0.0652 | 0.24 | 1.15 |

horseradish peroxidase activity. Several exposures of each blot were developed, and bands corresponding to SH2 and BT2 protein subunits were quantified using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

## AGP Activity

The determination of AGP activity was performed as described by Hannah and Nelson (1975). Activity values presented in Table I represent amounts relative to the standard in the presence of 20 mM 3-phosphoglyceric acid.

## Isolation of Nuclei and Run-On Analysis

Nuclei were obtained from kernels that had been quick-frozen in liquid $N_2$. The nuclei were isolated, and the run-on transcription assays were performed according to the method of Walling et al. (1986). Quantification of nuclei was by UV spectroscopy. Labeled transcripts were isolated according to the method of Manley et al. (1983). Addition of 2 $\mu$g/mL $\alpha$-amanitin decreased incorporation below detection. Hybridizations were with 7.5 $\mu$g of each plasmid on Southern blots using hybridization and washing conditions for the DNA and RNA blots. Blots were quantified with a Molecular Dynamics Phosphorimager. Values were standardized to the rDNA signals.

## In Vitro Kernel Culture

Blocks of W64A × 182E $F_2$ kernels were isolated at 22 dpp from the field-grown maize and surface sterilized according to the methods of Gengenbach (1977). They were placed on filter paper in Petri plates moistened with filter-sterilized Suc or mannitol solutions. Plates were incubated for 4 d at 22°C in the dark. Kernels were quick-frozen, RNA was isolated, and the transcript amounts were determined as described above.

# RESULTS AND DISCUSSION

## Expression of Starch Synthetic Genes in Selected Starch Mutants

Previously we reported that the Sh2 transcript was elevated in *bt2-* mutants and the Bt2 transcript was elevated in *sh2-* mutants (Bae et al., 1990). Since *Sh2* and *Bt2* encode subunits of one enzyme, AGP, and because the genes likely share a common evolutionary origin (Bae et al., 1990; Bhave et al., 1990), we entertained the idea that the data from the mutants were uncovering a form of end-product feedback control. Alternatively, the elevated transcripts could reflect a more global effect, perhaps related to changes in amounts of a metabolite involved in starch synthesis. To determine whether the elevation of Sh2 and Bt2 transcripts was confined to *sh2-* and *bt2-* mutants, we assayed transcript and protein amounts in additional mutants that affect starch content (Fig. 1).

In agreement with previous data, the relative amount of Sh2 transcript is increased in *bt2-* mutants and the Bt2 transcript is increased in *sh2-* mutants (Fig. 1A). That the increase is not confined to AGP structural genes is also shown in Figure 1. The relative amount of Sh2, Bt2, and Sh1 transcripts present in *sh2-*, *sh1-*, *bt1-*, and *bt2-* mutants at 22 dpp was analyzed by the quantification of northern blots. The largest increases in Bt2 transcript were in the *sh2-* and *bt1-* mutants, whereas no increase in *sh1-* was observed. Likewise, the Sh2 transcript was increased in both the *bt2-* and *bt1-* mutants, but in *sh1-* it was relatively unaffected. There was a noticeable but small increase in the amount of the Sh1 transcript in *bt1-* and *bt2-* mutants. Since both the Sh2 and Bt2 transcripts were increased in the *bt1-* mutant and *Bt1* is the structural gene for an adenylate carrier (Sullivan et al., 1991), we conclude that the signal causing the increased Sh2 and Bt2 transcripts is not the loss of these transcripts and functional AGP activity but rather is related to some other change in the starch biosynthetic pathway.

Additionally, we asked whether the increased transcript content led to an increased amount of protein. The use of SH2- and BT2-specific antibodies showed that SH2 protein

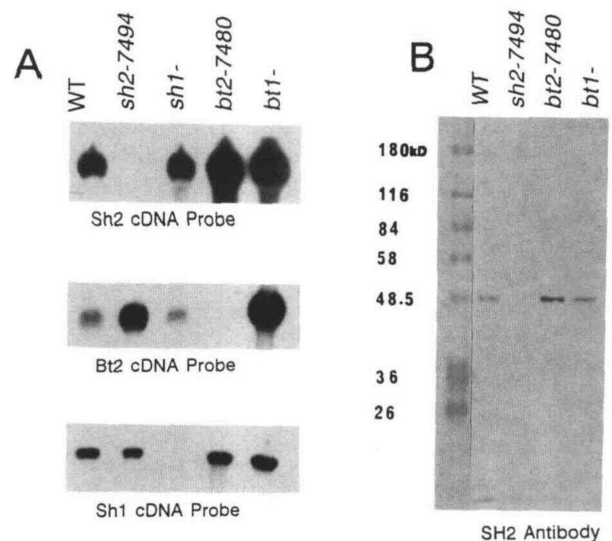



**Figure 1.** Interaction of starch biosynthetic mutants. A, Compilation of three RNA gel blots each with five lanes. The genotypes and cDNA probes are as listed. For each genotype, 7.5 $\mu$g of total RNA were loaded. B, Western blot of the same genotypes with 10 $\mu$g of total buffer-soluble protein from 22-dpp kernels loaded in each lane. The western blot was probed with the SH2 antibody. The wild type (WT) utilized was standard W64A × 182E $F_2$ kernels.

increased in both the *bt1*- and *bt2*- mutants (Fig. 1B), and the BT2 protein increased in *sh2*- and *bt1*- mutants (data not shown). These data point to Sh2 and Bt2 transcript content as one (and perhaps the major) limiting step in the synthesis of the two AGP subunits and are consistent with the observed increase in enzymic activity associated with an increase in the number of functional *Sh2*- and *Bt2*- alleles (Hannah and Nelson, 1975).

The Sh2 and Bt2 transcripts barely increased in the *sh1*- mutant. The product of *sh1*, Suc synthase, comes early in the starch biosynthetic pathway, whereas the proteins of the *sh2*, *bt2*, and *bt1* genes function much later. Possibly, elevation in a metabolite following cleavage of Suc, but before the synthesis of ADP-Glc, is involved in the response. However, *sh1*- mutants differ from most *sh2*-, *bt2*-, and *bt1*- mutants in that the latter are associated with a more severe block in the pathway, resulting in lower starch content and greater increases in soluble sugars. Conceivably, then, the differences noted in Figure 1 reflect differences in the severity of the genetic block.

To address this question, we examined two *sh2*- mutant alleles of varying severity. Although most *sh2* mutants are phenotypically indistinguishable, the mutant allele *sh2-I*, isolated by M. Neuffer, leads to a phenotype intermediate between wild type and the standard *sh2-R* phenotype (Hannah et al., 1993). As a measurement of this, mature backcross-3 kernels from segregating ears of the genotypes, *sh2-I/sh2-I/sh2-R* and *sh2-R/sh2-R/sh2-R* were weighed. The *sh2-I*-containing kernels averaged 0.140 g/kernel, whereas the other class averaged 0.088 g/kernel. Furthermore, relative amounts of AGP activity in the endosperm and embryo and seed weights at 22 dpp were measured (Table I) in *sh2-R* and *sh2-I* as well as in the phenotypically severe *bt2*- mutant, *bt2-B*. As expected, the *sh2-I* mutant has higher AGP activity in the endosperm relative to the other AGP mutants. Embryo AGP activity was not reduced in these mutants and may in fact be increased relative to the standard W64A × 182E $F_2$ kernels used.

The two mutant *sh2*- alleles also differ in the extent of elevation of the Bt2 transcript as well as the transcript of the endosperm-specific, starch synthetic gene *Wx1* (Table II). We surveyed a series of 10 *sh2*- mutants and 8 *bt2*- mutants for Bt2 and Sh2 transcript amounts, respectively (data not shown). In agreement with the pattern seen with *sh2-R* and *sh2-I*, phenotypically less severe mutants conditioned smaller increases in the transcript of the nonallelic gene. When these data are considered together, it appears that the extent of increase in the nonmutant transcripts is related to the severity of the block in the starch biosynthetic pathway rather than the actual position of the biochemical lesion in the pathway, a conclusion also supported by data presented in a later section.

To determine whether elevated transcripts are in kernel components other than the endosperm, transcripts in *sh2-R* and *sh2-I* embryos and endosperms were determined (Table II). Although small increases were noted in embryo transcripts, much larger increases in transcripts were noted in the endosperm. This was expected since the genes examined are endosperm specific and are involved in the production of endosperm storage compounds.

The source of the transcript increase in *sh2-I*, *sh2-R*, and *bt2-B* was addressed with run-on transcription assays (Table III). Transcription rates of endosperm-specific starch biosynthetic genes were elevated and were comparable to the increases in the steady-state levels of transcripts. The increased sh2-I transcript relative to standard W64A × 182E $F_2$ kernels likely represents part of the response of starch genes to feedback control. Transcription of *sh2-R* was undetectable. *sh2-R* has an insertion in the 5′ region of the transcribed region (J.R. Shaw, M.J. Giroux, and L.C. Hannah, unpublished data). We conclude that the elevation in the nonmutant transcripts is a consequence of increased transcription rather than an effect at the posttranscriptional level.

## Transcript Increases Exist in Additional Starch Biosynthetic Mutants

To extend these analyses to other mutations affecting storage products of the maize endosperm, isogeneic lines containing a number of endosperm-specific mutations were examined. The W64A isogeneic lines utilized were wild type or contained a single *sh1*-, *sh2*-, *bt1*-, *bt2*-, or *o2*- mutation. A W64A isoline containing the triple mutant *ae1*- *du1*- *wx1*- was also used. Table IV presents Sh1, Sh2, Bt2, Wx1, S zein, and L zein transcript amounts at early (14 dpp), middle (22 dpp), and late (30 dpp) stages of development. Representative northern blots of 22-dpp kernels are shown in Figure 2. The middle stage (22 dpp) is recognized as the peak of starch

**Table II.** *Bt2, S zein, Agp2, and Wx1 transcript content of the same sh2- and bt2- mutants shown in Table I and embryo (Embr)- and endosperm (Endo)-specific transcripts in selected sh2- mutants at 22 dpp*

The transcripts were measured from northern blots containing 7.5 μg of total RNA at 22 dpp and expressed relative to our standard wild type

| Transcript | Relative Steady-State Transcript Amount | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | WT | *sh2-I* | *sh2-R* | *bt2-B* | WT | | *sh2-I* | | *sh2-R* | |
| | | | | | Endo | Embr | Endo | Embr | Endo | Embr |
| Bt2 | 1.00 | 3.97 | 6.91 | 0.43 | 1.00 | 1.00 | 3.96 | 1.86 | 6.55 | 1.28 |
| S zein | 1.00 | 0.73 | 0.74 | 0.85 | 1.00 | 1.00 | 0.92 | 1.04 | 0.67 | 0.92 |
| Agp2 | 1.00 | 0.83 | 1.89 | 1.28 | 1.00 | 1.00 | 1.25 | 1.14 | 1.81 | 1.34 |
| Wx | 1.00 | 1.27 | 4.09 | 2.91 | 1.00 | 1.00 | 1.79 | 1.44 | 3.70 | 1.96 |

**Table III.** *Transcription rates of endosperm-specific genes in selected sh2- and bt2- mutants*

Nuclei were extracted from intact kernels at 22 dpp. Transcription rates were expressed relative to standard W64A × 182E $F_2$. Data are the averages of two or more experiments. SD values ranged from 1 to 23%.

| Transcript | Relative Transcription Rates | | | |
|---|---|---|---|---|
| | WT | *sh2-I* | *sh2-R* | *bt2-B* |
| Sh1 | 1.00 | 0.93 | 1.10 | 1.33 |
| Sh2 | 1.00 | 2.62 | 0 | 4.82 |
| Bt2 | 1.00 | 7.64 | 7.08 | 0 |
| Wx1 | 1.00 | 3.54 | 3.28 | 6.08 |
| S zein | 1.00 | 1.06 | 0.91 | 0.81 |

biosynthesis (Ingle et al., 1965; Tsai et al., 1970). Mutant transcript content is expressed as a proportion of that in the standard W64A kernels in which transcripts of the starch synthetic genes, per total RNA, are highest at 14 dpp, remain high, and then rapidly decline at approximately 25 dpp (data not shown). Amounts at 30 dpp represent only 5 to 15% of those seen at 14 dpp.

If the data are expressed on a protein or endosperm basis, a peak at 22 dpp occurred with most transcripts. Furthermore, AGP and Sh2 and Bt2 transcript developmental profiles were virtually superimposable in wild type when the data were expressed on a kernel or protein basis (data not shown). The decline in Sh2 and Bt2 transcripts occurred more rapidly than that reported recently by Prioul et al. (1994); however, this most likely represents differences in growing conditions.

At 14 dpp, the greatest increases in the endosperm-specific transcripts were in *bt1-*, *bt2-*, and *ae1- du1- wx1-* mutants (Table IV). These mutants severely block the starch biosynthetic pathway. The Bt2 and Wx1 transcripts were the most highly elevated at this stage of development. The increases relative to wild type were a maximum of 478% in the *bt1-* mutant for the Bt2 transcript. Sh1, S zein, and L zein transcripts decreased or were only moderately increased.

The increases at 14 dpp were accentuated at 22 dpp (Table IV). The greatest increases (more than 5-fold) were again observed in the mutants in which starch synthesis is severely blocked. At 30 dpp, this pattern was amplified (Table IV). Increases of 10-fold or more occurred in the *sh2-*, *bt1-*, *bt2-*, *o2-*, and the *ae1- du1- wx1-* mutants. The Sh2 and Wx1 transcripts were most affected.

A surprising observation was the increased accumulation of the starch synthetic transcripts in the *o2-* mutant. Increases of approximately 10-fold were noted in the Sh1 and Sh2 transcripts at 30 dpp. Since *O2* is involved in zein biosynthesis with no reported effects on starch biosynthesis, this result was unexpected. Since starch synthesis transcripts were altered in the *o2-* mutant, we asked whether zein transcripts might be altered in the starch mutants.

At 14 dpp, there were small increases in S and L zein transcripts in *sh2-* (Table IV). Decreases in these transcripts relative to the standard W64A were observed in the remainder of the mutants. At 22 dpp, there were increases in the zein transcripts in most starch mutants (Table IV). The greatest increases were in *bt1-* and in *bt2-*. Greater than 10-fold increases occurred in *sh1-*, *wx1-*, and *ae1-du1-wx1-* at 30 dpp in S and L zein transcripts (Table IV). The lowest overall increases were in *bt1-* and *o2-*. Large increases in S zein transcript were not unexpected since *O2* affects mainly the production of 22-kD (L) zein proteins.

The expression of genes not known to affect the production of storage products in the maize endosperm were also followed in the mutants. Clones of the genes, *agp1* and *agp2*, embryo counterparts of *sh2* and *bt2*, respectively (Giroux and Hannah, 1994), and *sus1*, which encodes Suc synthase primarily in the embryo (McCarty et al., 1986), were used to compare transcripts in 22- and 30-dpp kernels (Table IV). Although there were some increases in these transcripts at 22 dpp (e.g. a 4-fold increase in Agp1 transcript in *ae1- du1- wx1-*), the increase in transcripts conditioned by these mutants was generally less than that observed for endosperm-specific storage product genes. However, increases in the embryo genes would be expected if they were induced by mechanisms that affect the kernel as a whole and that were not simply endosperm specific. Although the *sh2* and *bt2* genes are endosperm specific and do not reduce the AGP activity in the embryo, sugar concentrations are elevated in mutant embryos. TB-A translocations were used to generate seed having a wild-type embryo but a *sh2-* mutant endosperm. Embryos of such seed had an elevated sugar content (C. Parera, D. Cantliffe, D.R. McCarty, and L.C. Hannah, unpublished data).

### SH2 and BT2 Proteins Are Not Elevated to the Same Extent as Their Transcripts in the Starch Mutants

Because of the magnitude of the increase in transcripts noted in the mutants conditioning severe blocks in starch synthesis at 30 dpp, we asked whether the cognate protein was also increased to the same extent. SH2 and BT2 proteins were measured in the isogeneic mutants at 14 and 30 dpp (Fig. 3). Although the proteins were elevated in some of the severely blocked starch mutants (*bt1-* and the triple mutant *ae1- du1- wx1-*) as well as *wx1-* and *du1-*, the increase was not nearly as great as noted for the transcripts. Mutants not increasing transcripts showed no increase in proteins. The approximate 2-fold increase in the amount of SH2 and BT2 protein in *bt1-* is in agreement with the reported elevated AGP activity in developing *bt1-* kernels (Doehlert and Kuo, 1990).

Interestingly, both BT2 and SH2 proteins were reduced in

**Table IV.** *Relative transcript amounts in endosperm-specific and nonendosperm-specific genes in W64A isogeneic lines at 14, 22, and 30 dpp*

Transcript amounts were quantified from northern blots prepared from 7.5 μg of total RNA at 14, 22, or 30 dpp. NA, Transcript amount was not determined. WT, Wild type. Data are the averages of two or more experiments. SD values ranged from 2 to 26%.

| Transcript | Line | | | | | | |
|---|---|---|---|---|---|---|---|
| | WT | *sh1-* | *sh2-* | *bt1-* | *bt2-* | *o2-* | *ae1- du1- wx1-* |
| | | | | *14 dpp* | | | |
| Sh1 | 1.00 | 0.04 | 1.06 | 0.56 | 0.84 | 0.50 | 0.62 |
| Sh2 | 1.00 | 1.11 | 0.11 | 1.88 | 3.09 | 1.11 | 1.74 |
| Bt2 | 1.00 | 1.86 | 2.16 | 4.78 | 0.21 | 1.94 | 3.54 |
| Wx1 | 1.00 | 0.64 | 1.23 | 2.92 | 2.73 | 1.10 | 0.15 |
| S zein | 1.00 | 0.78 | 1.25 | 0.81 | 0.71 | 0.29 | 0.60 |
| L zein | 1.00 | 0.70 | 1.52 | 0.76 | 0.59 | 0.13 | 0.37 |
| | | | | *22 dpp* | | | |
| Sh1 | 1.00 | 0.28 | 2.30 | 3.43 | 4.72 | 2.85 | 2.37 |
| Sh2 | 1.00 | 1.00 | 0.19 | 5.40 | 6.72 | 2.46 | 4.74 |
| Bt2 | 1.00 | 2.32 | 5.16 | 4.27 | 0.25 | 2.40 | 5.51 |
| Wx1 | 1.00 | 2.18 | 5.43 | 6.56 | 8.36 | 1.50 | 0.30 |
| S zein | 1.00 | 2.29 | 1.60 | 3.06 | 3.08 | 0.50 | 1.86 |
| L zein | 1.00 | 1.07 | 0.67 | 1.36 | 1.49 | 0.04 | 0.71 |
| Agp1 | 1.00 | 2.87 | 3.23 | 2.95 | 4.57 | 3.09 | 4.22 |
| Agp2 | 1.00 | 1.99 | 2.10 | 1.81 | 2.84 | 1.96 | 2.40 |
| Sus | 1.00 | 1.05 | 0.95 | 1.22 | 1.33 | 1.17 | 1.37 |
| | | | | *30 dpp* | | | |
| Sh1 | 1.00 | 0.65 | 3.82 | 5.10 | 7.73 | 9.12 | 9.98 |
| Sh2 | 1.00 | 9.28 | 0.52 | 10.45 | 20.98 | 10.14 | 33.63 |
| Bt2 | 1.00 | 4.61 | 4.91 | 3.80 | 0.12 | 2.16 | 9.11 |
| Wx1 | 1.00 | 6.93 | 12.19 | 4.96 | 16.09 | 5.18 | 2.05 |
| S zein | 1.00 | 19.00 | 7.94 | 4.01 | 11.99 | 9.2 | 18.53 |
| L zein | 1.00 | 25.90 | 6.00 | 5.35 | 8.05 | 1.39 | 13.20 |
| Agp1 | 1.00 | 2.76 | NA | 2.67 | 2.12 | 3.73 | 4.34 |
| Agp2 | 1.00 | 1.85 | NA | 1.32 | 1.35 | 1.90 | 2.15 |
| Sus1 | 1.00 | 2.02 | NA | 1.07 | 1.52 | 1.09 | 1.91 |

amount in the *bt2-* and *sh2-* mutants at 30 dpp. The BT2 protein is, in fact, synthesized in *sh2-* and the SH2 protein is synthesized in *bt2-* (Fig. 3). At 14 dpp, the BT2 protein in *sh2-* is comparable (83%) to wild type and the SH2 protein in *bt2-* is comparable (117%) to wild type. Their abundance at 14 dpp but decline at 30 dpp, relative to that in wild type and the other mutants, suggests that the SH2 and BT2 proteins are more susceptible to degradation when the formation of a SH2/BT2 polymer does not occur.

## Transcription Increases Coincide with Increased Transcripts

Nuclei isolated from standard W64A, *bt1-*, *bt2-*, and *wx1-* mutant kernels at 30 dpp were used in nuclear run-on assays, and transcription of the *Sh1, Sh2, Bt2, Wx1,* and *S zein* genes were monitored. Figure 4 presents transcriptional activity in the mutants relative to the standard W64A. The increases are of the same magnitude as the increased steady-state transcript content. Increased transcription rates apparently account for the increase in steady-state transcript, as has been shown for hordein B and C genes (Sorenson et al., 1989).

## Influence of Suc and Mannitol on Transcript Amounts

The increased transcripts in the starch-deficient mutants could be due to a buildup in a metabolite(s) in the synthesis of starch or to lower starch content. In an attempt to distinguish between these possibilities, an in vitro kernel development procedure was used to alter the amount of Suc entering wild-type seed. If the response in the mutants is due to deficient starch content, we expect that transcripts would decrease as Suc in the growth medium increases from suboptimal to optimal concentrations. Conversely, if the mutant response is due to elevated sugar concentrations, a positive correlation between transcript amounts and sugars would be expected.

Cobb and Hannah (1983) reported that maize kernels grown in vitro develop normally, resemble field-grown kernels, and develop to maturity at 30 to 35 dpp when supplied with relatively high Suc. In subsequent studies, Cobb et al. (1988) showed that lower Suc concentrations (0.06–0.3 M) led to suboptimal growth, as judged by lowered kernel weight.

Table V shows the resulting transcript content when seeds

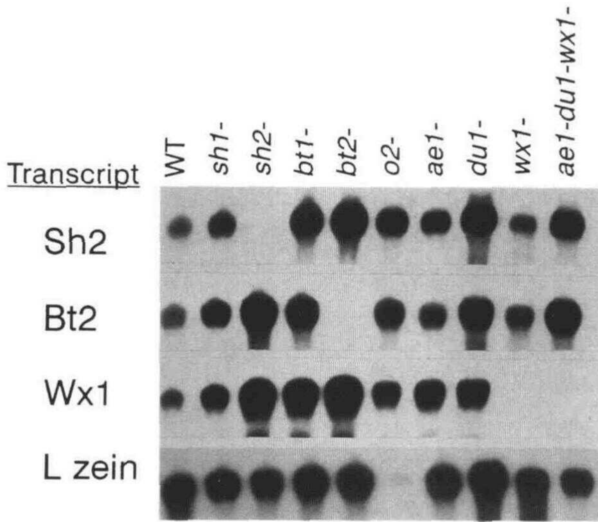



**Figure 2.** Representative northern blots of isogeneic developing kernels in a W64A background harvested at 22 dpp. These blots are representative of the data presented in Table IV. Area shown is the region of hybridization for each of the listed transcripts.

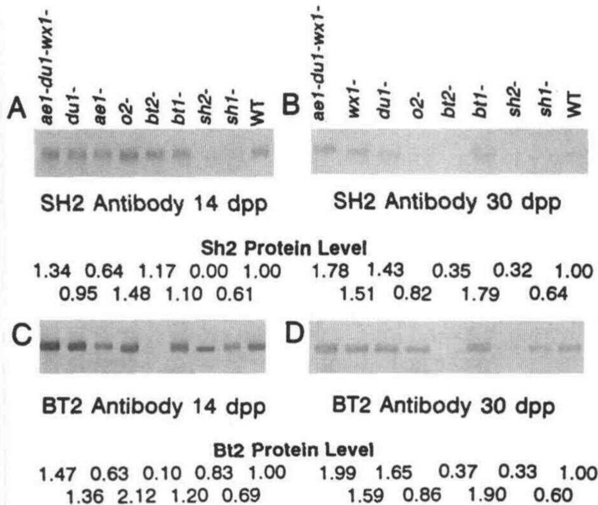
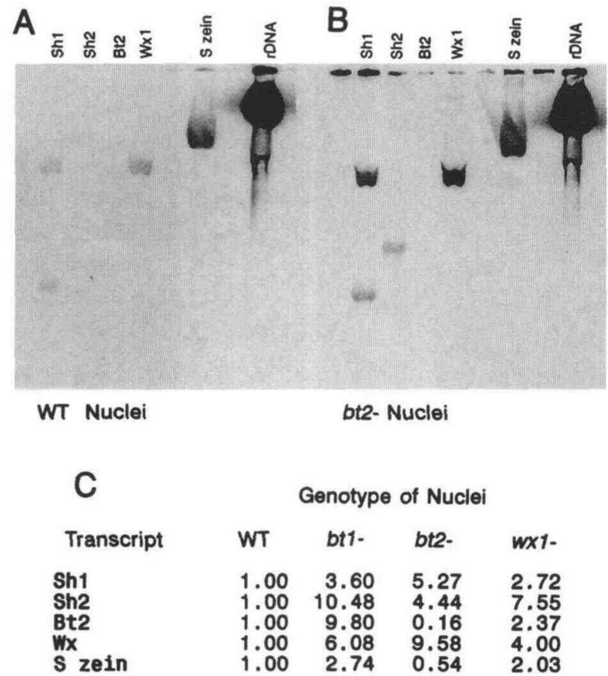



**Figure 3.** Western analysis of the SH2 and BT2 proteins in isogeneic lines at 14 and 30 dpp. The relative protein content of SH2 and BT2 was determined in the W64A genotypes as listed for each figure. Total buffer-soluble protein (20 $\mu$g) was loaded for each genotype in A, B, C, and D. The SH2 antibody was used in A and B and the BT2 antibody was used in C and D. A and C represent protein samples from 14-dpp kernels and B and D represent protein samples from 30-dpp kernels. Data are the average of two experiments and are expressed relative to the standard W64A (WT). SD values ranged from 0 to 21%.




| Transcript | Genotype of Nuclei | | | |
|---|---|---|---|---|
| | WT | *bt1-* | *bt2-* | *wx1-* |
| Sh1 | 1.00 | 3.60 | 5.27 | 2.72 |
| Sh2 | 1.00 | 10.48 | 4.44 | 7.55 |
| Bt2 | 1.00 | 9.80 | 0.16 | 2.37 |
| Wx | 1.00 | 6.08 | 9.58 | 4.00 |
| S zein | 1.00 | 2.74 | 0.54 | 2.03 |

**Figure 4.** Relative run-on transcription at 30 dpp in isogeneic nuclei. A and B are Southern blots with lanes containing Sh1, Sh2, Bt2, Wx1, and S zein cDNAs. Blot A was probed with labeled transcripts from wild-type nuclei, and blot B was probed with transcripts from *bt2−* nuclei. Blots probed with RNA from *bt1−* and *wx1−* are not shown. An rDNA clone was used as a control for the total amount of hybridization and as a marker for correct film exposures. The counted values from a Molecular Dynamics Phosphorimager were corrected based on the rDNA value for each blot, and final corrected transcription rate values are presented in C. Data presented in C are averages of two or more experiments. SD values ranged from 10 to 28%.

developed on varying concentrations of Suc or mannitol. Sh2 and Bt2 transcripts increased as the Suc in the growth medium was elevated, whereas the Sh1 and S zein transcripts were much less responsive. This differential response of the transcripts to alterations in Suc in the growth medium mimics the alterations in transcripts of the high sugar/low starch mutants (*sh2-*, *bt2-*, *bt1-*, and the triple mutant *ae1- du1- wx1-*). Furthermore, since the low concentrations of Suc in the growth medium give rise to reduced seed weight (Cobb et al., 1988) and presumably lowered starch content, the signal leading to the elevation in transcripts may involve an increase in sugars rather than the reduced starch content. The transcripts most increased, Sh2, Bt2, and Wx, were also those most involved in the type and amount of starch produced. This argues that genes directly involved in producing storage products in the endosperm are most sensitive to an increase in metabolically active sugars. Sh2 and Bt2 transcripts were also modulated by varying mannitol in the growth medium. Although the pattern of change was less pronounced than that seen with Suc, the alteration in transcript amounts may be a response to altered osmotic conditions rather than a direct effect of Suc.

Sugar effects have been reported for other genes as well.

**Table V.** *Relative Sh1, Sh2, Bt2, and S zein transcripts in wild-type kernels grown on different concentrations of Suc or mannitol*

Sh1, Sh2, Bt2, and S zein transcripts were determined in W64A kernels at 22 and 26 dpp. Kernels that were 22 dpp were isolated and incubated for 4 d on 0.06, 0.3, 0.6, and 0.9 M Suc or mannitol. The transcript contents relative to the levels in the 22-dpp field material were determined from RNA gel blots containing 7.5 μg of total RNA. The blots were probed with Sh1, Sh2, Bt2, and S zein cDNAs. The transcripts are expressed relative to the field material at 22 dpp. SD values ranged from 0 to 10%.

| Treatment (M) | Transcript | | | |
|---|---|---|---|---|
| | Sh1 | Sh2 | Bt2 | S zein |
| Suc | | | | |
| 0.06 | 0.53 | 0.34 | 0.25 | 0.44 |
| 0.30 | 0.74 | 0.66 | 0.62 | 0.54 |
| 0.60 | 0.72 | 0.91 | 1.01 | 0.37 |
| 0.90 | 0.42 | 0.91 | 0.77 | 0.46 |
| Mannitol | | | | |
| 0.06 | 0.66 | 0.52 | 0.37 | 0.47 |
| 0.30 | 0.91 | 0.81 | 0.80 | 0.87 |
| 0.60 | 0.66 | 0.52 | 0.39 | 0.65 |
| 0.90 | 0.55 | 0.34 | 0.35 | 0.52 |
| Field 22 dpp | 1.00 | 1.00 | 1.00 | 1.00 |
| Field 26 dpp | 0.73 | 0.72 | 0.75 | 0.81 |

For example, the transcriptional activity of maize photosynthetic promoters is repressed by Suc, Glc, and acetate in maize protoplasts relative to a mannitol control (Sheen, 1990). More germane to our system, Koch et al. (1992) showed that *Sh1* and *Sus1* expression in maize root tips is modulated by sugar concentration, with each gene exhibiting a distinctive profile. Taken together, the evidence indicates that the effects of sugars may be related to the identity of the tissue as a source of or a sink for carbohydrates, as has been suggested by others. Genes involved in the production of Suc would be repressed by Suc and those involved in the production of starch from Suc would be stimulated. Such an explanation may explain part of the global results presented here in the starch and storage protein mutant backgrounds.

## Endosperm Storage Product Genes Are Coordinately Regulated

Coordinate gene expression is required for the successful development of any organism, including the maize kernel. Our data show that expression of many genes involved in endosperm development is sensitive to perturbations in developmental or biochemical processes seemingly not directly related to the affected gene. We show that transcript accumulation of genes involved in starch and storage protein synthesis is elevated when the synthesis of these end products is impaired. The alteration is at the transcriptional level, is dependent on the severity of the lesion, and does not alter the expression of the affected genes to the same degree. The pronounced elevation in Sh2 and Bt2 transcripts is associated with increases in the cognate proteins, albeit of a much lesser magnitude. The genetic lesion, at least for starch synthesis, can be simulated by alterations in the concentration of Suc fed to the kernel. Elevation in sugars or alteration in osmotic conditions, rather than the depletion in starch, apparently is involved in the signal transduction pathway.

The identification of the primary signal triggering this response awaits further investigation. Seemingly, the primary signal is not Suc, since transcripts are altered in mutants (e.g. *o2* and *wx*) not known to significantly alter sugar content. It is interesting to note that *o2*- kernels dry down more slowly than their wild-type counterparts on segregating and otherwise isogeneic developing ears (M. Lopes and B. Larkins, personal communication). Slower kernel dry-down is a well-known pleiotropic effect of the starch mutants, presumably because of the ability of the sugars to increase the moisture-holding capacity. Perhaps transcription of these storage-product genes is affected by the osmotic potential of the endosperm.

Although zein transcripts are elevated in the severely blocked starch mutants, zein protein is greatly reduced (Barbosa and Glover, 1978). Clearly, this reduction in zein is not due to a lack of zein transcripts. Furthermore, the increased transcripts of the starch synthetic genes in *o2*- may be related to the increase seen in the non-zein proteins.

Although SH2 and BT2 proteins are elevated in some mutants primarily at the latter stages of development, the extent of the increase is much less than that noted for their transcripts. This result is unexpected, since AGP activity shows a linear increase with the number of functional *Sh2* or *Bt2* alleles (Hannah and Nelson, 1975). Collectively, these data point to mechanisms regulating protein amount that are, or can become, independent of transcript abundance. This is similar to a case described for the *Sh1* gene in which transcripts increase in response to anaerobiosis without a concomitant increase in protein (Talliercio and Chourey, 1989). However, it contrasts with the anaerobic induction of Suc synthase transcription and translation in rice (Ricard et al., 1990).

Aside from the complexity mentioned above, our data also suggest that the rates of SH2 or BT2 protein turnover are dependent on the presence of a BT2 or SH2 protein, respectively. The SH2 protein is present in wild-type amounts in

14-dpp *bt2-* seeds; yet in 30-dpp *bt2-* kernels, it is greatly reduced relative to wild type and the other mutants. The identical pattern was seen with the BT2 protein in *sh2-* mutants. These observations are reminiscent of an *Arabidopsis* mutant (Lin et al., 1988) that lacks both subunits of AGP. Although this mutation may lie in a regulatory gene involved in AGP synthesis, as the authors suggest, it seems equally plausible that the mutation is in a structural gene and the subunit of the nonallelic, wild-type gene is unstable in the absence of the other subunit.

## ACKNOWLEDGMENTS

The Waxy1 cDNA probe was generously provided by Susan Wessler (University of Georgia). Quantification of blots, both with the AMBIS and the Molecular Dynamics Phosphorimager and scanning densitometer, was done in a facility of the Interdisciplinary Center for Biotechnology Research (University of Florida).

Received March 23, 1994; accepted June 29, 1994.
Copyright Clearance Center: 0032-0889/94/106/0713/10.

## LITERATURE CITED

**Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K** (1987) Current Protocols in Molecular Biology. Greene Publishing and Wiley-Interscience, New York

**Bae JM, Giroux M, Hannah L** (1990) Cloning and characterization of the *brittle-2* gene of maize. Maydica **35:** 317–322

**Barbosa HM, Glover DV** (1978) Genes and gene interactions affecting protein and lysine content in the endosperm of maize. Braz J Genet **1:** 29–39

**Beavis W, Berlyn M, Burr B, Chandler V, Coe E, Nelson O** (1993) A standard for maize genetics nomenclature. Maize Genet Newslett **67:** 171–173

**Bhave MR, Lawrence S, Barton C, Hannah LC** (1990) Identification and molecular characterization of *shrunken-2* cDNA clones of maize. Plant Cell **2:** 581–588

**Boyer CD, Preiss J** (1978) Multiple forms of starch branching enzymes of maize: evidence of independent genetic control. Biochem Biophys Res Commun **80:** 169–175

**Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

**Chourey PS, Nelson OE** (1976) The enzymatic deficiency conditioned by the *shrunken-1* mutations in maize. Biochem Genet **14:** 1041–1055

**Church GM, Gilbert W** (1984) Genomic sequencing. Proc Natl Acad Sci USA **81:** 1991–1995

**Cobb BG, Hannah LC** (1983) Development of wild type, *shrunken-1*, and *shrunken-2* maize kernels grown *in vitro*. Theor Appl Genet **65:** 47–51

**Cobb BG, Hole DJ, Smith JD, Kent MW** (1988). The effects of modifying sucrose concentration on the development of maize kernels grown *in vitro*. Ann Bot **62:** 265–270

**Creech RG** (1965) Genetic control of carbohydrate synthesis in maize endosperm. Genetics **52:** 1175–1186

**Doehlert DC, Kuo TM** (1990) Sugar metabolism in developing kernels of starch deficient endosperm mutants of maize. Plant Physiol **92:** 990–994

**Gengenbach BG** (1977) Development of maize caryopses resulting from *in-vitro* pollination. Planta **134:** 91–93

**Giroux MJ, Hannah LC** (1994) Expression of ADP-glucose pyrophosphorylase genes in shrunken2 and brittle2 mutants of maize. Mol Gen Genet **431:** 400–408

**Habben JE, Kirleis AW, Larkins BA** (1993) The origin of lysine-containing proteins in *opaque-2* maize endosperm. Plant Mol Biol **23:** 825–838

**Hannah LC, Giroux M, Boyer CD** (1993) Biotechnological modification for sweet corn and maize improvement. Sci Hortic **55:** 177–197

**Hannah LC, Nelson OE Jr** (1975) Characterization of adenosine diphosphate glucose pyrophosphorylases from developing maize seeds. Plant Physiol **55:** 297–302

**Hannah LC, Nelson OE Jr** (1976) Characterization of ADP- glucose pyrophosphorylase from *shrunken-2* and *brittle-2* mutants of maize. Biochem Genet **14:** 547–560

**Ingle J, Beitz D, Hageman RH** (1965) Changes in composition during development and maturation of maize seeds. Plant Physiol **40:** 835–839

**Koch KE, Nolte KD, Duke ER, McCarty DR, Avigne WT** (1992) Sugar levels modulate differential expression of maize sucrose synthase genes. Plant Cell **4:** 59–69

**Laemmli V** (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4. Nature **227:** 680–685

**Langridge P, Pintor-Toro JA, Feix G** (1982) Transcriptional effects of the opaque-2 mutation of *Zea mays* L. Planta **156:** 166–170

**Lin T-P, Caspar T, Somerville C, Preiss J** (1988) Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* lacking ADPglucose pyrophosphorylase activity. Plant Physiol **86:** 1131–1135

**Ma Y, Nelson OE** (1975) Amino acid composition and storage proteins in two new high-lysine mutants in maize. Cereal Chem **52:** 412–418

**Maniatis T, Fritsch EF, Sambrook J** (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

**Manley JL, Fire A, Samuels M, Sharp PA** (1983) *In vitro* transcription: whole-cell extract. Methods Enzymol **101:** 568–582

**McCarty DR** (1986) A simple method for extraction of RNA from maize tissues. Maize Genet Newslett **60:** 61

**McCarty DR, Shaw JR, Hannah LC** (1986) The cloning, genetic mapping, and expression of the constitutive sucrose synthase locus of maize. Proc Natl Acad Sci USA **83:** 9099–9103

**Mertz ET, Bates LS, Nelson OE Jr** (1964) Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science **145:** 279–280

**Muller-Rober BT, Kobmann J, Hannah LC, Willmitzer L, Sonnewald U** (1990) One of two different ADP-glucose pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose. Mol Gen Genet **224:** 136–146

**Nelson OE, Mertz ET, Bates LS** (1965) Second mutant gene affecting the amino acid pattern of maize endosperm proteins. Science **150:** 1469–1470

**Nelson OE, Rines HW** (1962) The enzymatic deficiency in the waxy mutant of maize. Biochem Biophys Res Commun **9:** 297–300

**Prioul J-L, Jeannette E, Reyss A, Nicole G, Giroux M, Hannah LC, Causse M** (1994) Expression of ADP-glucose pyrophosphorylase in maize (*Zea mays* L.) grain and source leaf during grain filling. Plant Physiol **104:** 179–187

**Ricard B, Rivoal J, Spiteri A, Pradet A** (1990) Anaerobic stress induces the transcription and translation of sucrose synthase in rice. Plant Physiol **95:** 669–674

**Schmidt RJ, Burr FA, Aukerman MJ, Burr B** (1990) Maize regulatory gene *opaque-2* encodes a protein with a "leucine-zipper" motif that binds to zein DNA. Proc Natl Acad Sci USA **87:** 46–50

**Sheen J** (1990) Metabolic repression of transcription in higher plants. Plant Cell **2:** 1027–1038

**Sorenson MB, Cameron-Mills V, Brandt A** (1989) Transcriptional and post-transcriptional regulation of gene expression in developing barley endosperm. Mol Gen Genet **217:** 195–201

**Sullivan TD, Strelow LI, Illingworth CA, Phillips RL, Nelson OE Jr** (1991) The maize *brittle-1* locus: molecular characterization based on DNA clones isolated using the *dSpm*-tagged *brittle-1-mutable* allele. Plant Cell **3:** 1337–1348

**Talliercio EW, Chourey PS** (1989) Post-transcriptional control of sucrose synthase expression in anaerobic seedlings of maize. Plant Physiol **90:** 1359–1364

**Tsai CY, Larkins BA, Glover DV** (1978) Interactions of the *opaque-2* gene with starch-forming mutant genes on the synthesis of zein in maize endosperm. Biochem Genet **16:** 883–896

**Tsai CY, Salamini F, Nelson OE** (1970) Enzymes of carbohydrate metabolism in the developing endosperm of maize. Plant Physiol **46:** 299–306

**Walling L, Drews GN, Goldberg RB** (1986) Transcriptional and post-transcriptional regulation of soybean seed protein mRNA levels. Proc Natl Acad Sci USA **83:** 2123–2127