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Tissue-Specific Expression and Anaerobically Induced Posttranscriptional Modulation of Sucrose Synthase Genes in *Sorghum bicolor* M.[1]

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ABSTRACT

We have used antibodies directed against the two sucrose synthase (SS) isozymes, and the cDNA clones corresponding to the two nonallelic genes in maize to describe sorghum (*Sorghum bicolor*) SS genes and their expressions at protein and RNA levels. Western blot analyses have shown evidence of two SS isozymes, SS1 and SS2, in sorghum; these were similar, but not identical, to maize isozymes in size, charge, subunit composition, and epitope specificities against both monoclonal and polyclonal antibodies. Tissue-specific distributions of isozymes and genomic Southern hybridization data are consistent with a hypothesis that the SS1 and SS2 isozymes are encoded by two nonallelic genes, designated here as *Sus1* and *Sus2*, respectively. Northern blot hybridizations on root RNAs showed gene-specific transcript patterns and, as in maize, the SS2-specific transcripts were slightly larger than the SS1-specific transcripts. Interestingly, no difference in the size of the SS1 and SS2 polypeptides was detected. Anaerobic induction led to significant elevations in steady-state levels of both SS1 and SS2 transcripts, but there was no detectable increase in the levels of the SS proteins. Thus, both the SS genes in sorghum were significantly regulated at the posttranscriptional level; whereas in maize, only one of the two SS genes was affected in this fashion. Another difference between maize and sorghum SS isozymes was in endosperm-specific polymerization among the SS subunits. Unlike maize endosperm where only the two SS homotetramers are seen, sorghum endosperm showed five SS isozymes attributable to a random copolymerization of SS1 and SS2 subunits, presumably due to a simultaneous expression of both genes in the endosperm cells. Physiological and molecular bases of these differences between these two crop plant species remains to be elucidated.

The enzyme SS[2] (EC 2.4.1.13) plays an important role in energy metabolism by mobilizing sucrose into diverse pathways relating to metabolic, structural, and storage functions of the plant cell. The enzyme catalyzes reversible conversion of UDP and sucrose to UDP glucose and fructose and has been analyzed in a number of plant species. The earliest biochemical studies on SS are described in mung bean (11). The most detailed biochemical, physiological, and molecular genetic investigations on this enzyme, however, have been restricted to maize.

In maize, the two isozymes, SS1 and SS2, are encoded by the *Sh* and *Sus* genes, respectively (3, 4). The *Sh* locus on chromosome nine is highly expressed in developing endosperm. The loss of the SS1 protein, as in the case of the *shrunken* (*sh*) mutant, is associated with an approximately 40% reduction in the amount of endosperm starch (4) as well as an early cell degeneration which is unique to this mutant and is not a resultant effect of starch deficiency in the developing endosperm (8). Although the *Sus*-encoded SS2 isozyme is seen in nearly all tissues of the plant, a *Sus*-null mutant lacking the SS2 protein is not associated with any phenotypic change in the plant (7). The two SS isozymes are biochemically similar (3, 12) and show random copolymerization of subunits where both genes are expressed simultaneously in the same cell leading to the formation of SS heteromeric molecules, as seen in the extracts of young roots and shoots (6). The developing endosperm extracts, however, show only the two homomers (6); the lack of heteromers is mainly due to spatial separation in the expression of the two SS genes in this tissue (1). Such heterogeneity in terms of gene expression is indicative of differential *in vivo* roles of the two isozymes in maize endosperm. Another observation of much interest concerning the maize SS genes is the posttranscriptional regulation of the anaerobic induction of the *Sh* gene. It has been shown previously that anaerobic induction of the *Sh* gene at transcription level (22) is not accompanied by elevated levels of the SS1 protein (19, 24).

Sorghum (*Sorghum bicolor*) is a major crop plant and is evolutionarily closely related to maize. Conservations of chloroplast (9) and the nuclear genomes (15) between the two crop plants has been described. Recent analyses also show much conservation of linkage relationships, including the mapping of the *Sus1* gene to chromosome nine in sorghum (15). We describe here a comparative analysis of the SS expression in sorghum. Specifically, we sought to determine if the two rather unorthodox patterns of SS expression in maize, *viz.* tissue-specific polymerization of SS subunits and posttranscriptional control of anaerobic induction, were also seen in sorghum.

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[2] Abbreviations: SS, sucrose synthase; PCA, polyclonal antibody; MCA, monoclonal antibody; kb, kilobase; bp, base pair.

MATERIALS AND METHODS

Plant Material

These studies were done on a single cultivar of sorghum (*Sorghum bicolor*), NK 300. Seeds were germinated in flats of vermiculite and grown for 4 to 5 d in dark growth chamber. Seedlings for the anaerobic treatment were removed from vermiculite and submerged for 12 h in Tris HCl buffer (pH 7.5) or distilled water. The control (aerobic) population was left in the vermiculite for the duration. Following anaerobic treatment, seedlings from both environments were frozen in liquid nitrogen and stored at −70°C for the subsequent analyses. Several seedlings were used for protein analyses and the rest of the material was used for RNA preparations. Immature kernels were harvested approximately 2 weeks after anthesis, frozen in liquid nitrogen, and homogenized for protein analyses. Isolation of a cell suspension culture from NK 300 cultivar has been described previously (5). Five day-old cells, presumably in log phase of growth (5), were harvested, frozen in liquid nitrogen, and stored as above for protein and RNA analyses.

DNA Analysis

Sorghum genomic DNA was isolated from 5- to 7-d-old dark-grown seedlings as described (10). Approximately 15 μg of DNA was digested with the desired restriction enzyme according to the manufacturer's specifications (Bethesda Research Laboratories or Boehringer Mannheim). The digested samples were fractionated on 0.8% agarose gel and transferred to Nytran membrane and hybridized to ^{32}P-labeled cDNA probes corresponding to maize *Sh* or *Sus* gene (13). Blot hybridizations and washes were done as described (13).

Protein Analysis

Crude extracts from seedlings, immature endosperm, and embryo and cell suspension cultures were done as described previously (6). Protein measurements were made using Folin's phenol reagent (16). Samples were electrophoresed on a 4.5 to 7.0% linear gradient nondenaturing polyacrylamide gel for 15 to 18 h at 4 W constant power. Denaturing gels were done as described before (19). Western blots were done according to the method developed for maize SS proteins (6).

Monoclonal antibodies specific to maize SS1 and SS2 proteins were isolated through special assistance from the Hybridoma Core Laboratory (HCL) at the University of Florida. Partially purified preparations of developing maize endosperm extracts containing the two SS proteins were used in primary immunizations of mice. HCL assistance included immunization, isolation of spleen cells, and cell fusions using the standard technologies. ELISA screening was done using appropriate tissue extracts of maize to allow visualization of the two SS proteins. Polyclonal antibodies against maize SS proteins (12) were used to identify the specific SS proteins in the marker lanes. Production of ascites fluid and the purification of MCA was restricted to three clones, one each specific to SS1, SS2, and a third clone which recognized both the isozymes in Western blot analyses.

RNA Analysis

Total RNA was isolated by the method of Chirgwin *et al.* (2). Isolation of total polyribosomal RNA has been described previously (24). Poly $(A)^+$ RNA was isolated by two cycles of adsorption to elution from oligo(dt)-cellulose (17) and denatured and fractionated on 1% agarose-formaldehyde gels as described (13). RNA was transferred to Nytran membrane and prehybridized in 6 × SSC, 0.05 × BLOTTO, 50% formamide, and 100 μg/mL sheared and denatured salmon sperm DNA for 4 h at 42°C. Blot hybridizations and washes were done as described previously (25).

RESULTS

Genomic Hybridizations

Genomic Southern blot hybridizations using nearly full length maize *Sh* and *Sus* cDNA probes are shown in Figure 1. Unique major hybridizing fragments were identified by each gene probe in sorghum genomic DNA. The *Sh* probe showed a 3.2 kb *Eco*RI and a 9.4 kb *Bam*HI fragment; whereas the *Sus* probe identified an approximately 15.0 kb *Eco*RI and a 10.9 kb *Bam*HI fragment. No cross-homologies, evidenced by the lack of any hybridization to the *Sus* homologous sorghum fragments by the *Sh* probe or vice versa, were detected. It was remarkable that the pattern of hybridization with the heterologous DNA probes was similar to that seen in maize where the *Sh* probe does not detect any hybridizing

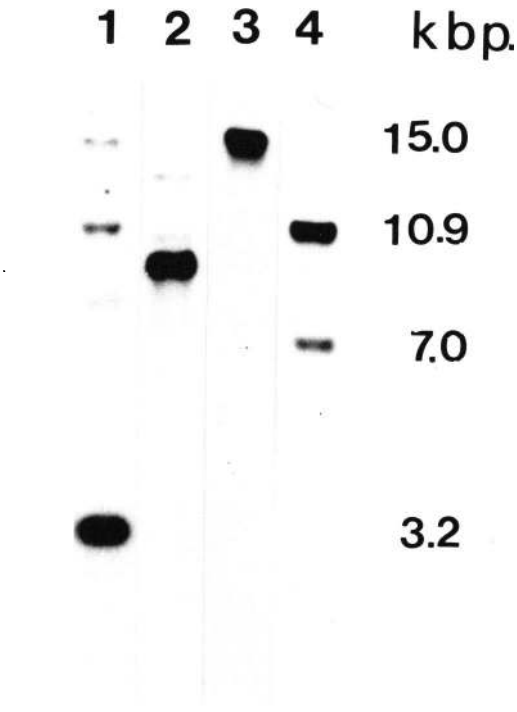

Figure 1. Genomic hybridizations of maize *Sh* (lanes 1–2) and *Sus* (lanes 3–4) cDNA probes to sorghum seedling DNA (15 μg per lane) restriction digested with *Eco*RI (lanes 1 and 3) and *Bam*H1 (lanes 2 and 4). Figures on the right represent size in kbp of the fragments detected by the probes.

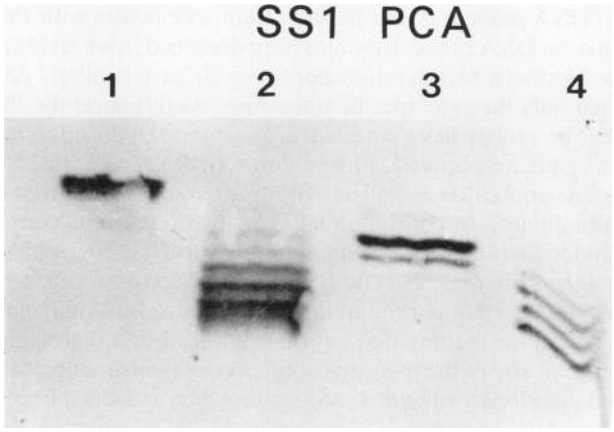

Figure 2. Nondenaturing Western blot showing sucrose synthase-specific protein bands in crude extracts from various tissues of sorghum (except lane 1) using the maize SS1 polyclonal antibody. Lane 1, developing endosperm extract, maize (2.5 μg), marks the SS1 protein; lanes 2 and 3 developing endosperm (25 μg) and embryo (25 μg), respectively; lane 4, roots from 4- to 5-d-old seedling (30 μg). Values in parentheses indicate the amount of total soluble protein.

bands corresponding to the *Sus* genomic fragments in a *Sh*-deletion strain (13).

SS Protein Blots

Immunostain reactions leading to SS specific protein bands on nondenaturing Western blots, using monospecific PCA against maize SS isozyme is shown in Figure 2. A total of five bands corresponding to five SS isozymes was seen in immature endosperm extracts (lane 2). Immature embryo extract exhibited a single slow major band (the least anodal) and a minor band which corresponded to the slowest of the five bands seen in other tissue extracts. Extracts from young roots showed four of the five isozymes seen in the endosperm extract, the fifth isozyme was the least abundant and is undetectable (however, see Fig. 6). A similar band pattern was seen using the maize SS2 PCA (data not shown). We have recently isolated and characterized MCA specific to maize SS1 and SS2 isozymes (see "Materials and Methods" for details). The general pattern of the SS bands on nondenaturing Western blots using the SS1 MCA (Fig. 3) was similar to that seen by the SS1 PCA (Fig. 2), except that the slowest migrating band, corresponding to the single band in embryo extract, exhibited significantly greater staining intensity with the SS2 MCA than the SS1 MCA (lane 3). Extracts from cell suspension culture showed a single major and a minor band using the SS1 MCA; these bands were, however, undetectable by the SS2 MCA. Interestingly, the multiple isozymes in sorghum endosperm and root extracts were undetectable by the SS2 MCA at the levels which were readily detectable by the SS1 MCA. We conclude that the more anodal or the faster migrating bands were predominantly due to the SS1 subunits and the less anodal or the slower migrating were composed of the SS2 subunits.

Quantitative differences in band intensities among the samples were better analyzable in denaturing Western blots (Fig. 4) as only a single band of identical electrophoretic mobility was seen in three of the four samples. Greater cross-reactivities of embryo (lane 2) and endosperm (lane 3) SS polypeptide to SS2 and SS1 MCAs, respectively, as judged by the color intensities of the bands was readily detectable. Endosperm extracts from both crop plants (lanes 1 and 3, respectively) showed similar staining intensities with the SS1 MCA at 1 μg level of the soluble protein in the gel. The highest level of signal for SS protein among sorghum samples was seen in the developing endosperm.

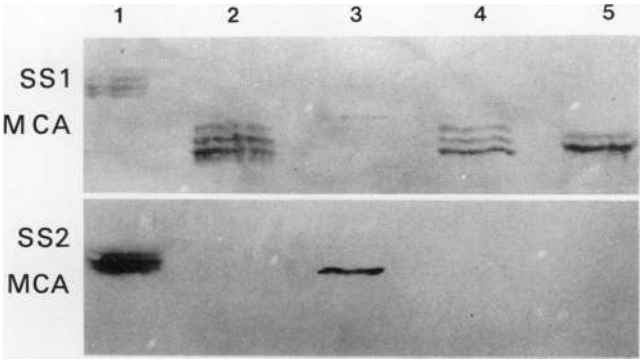

Figure 3. Nondenaturing Western blot showing sucrose synthase-specific protein bands in crude extracts from various tissues of sorghum (except lane 1) using the maize SS1- or SS2-specific monoclonal antibody, as indicated. Lane 1, maize root extract (30 μg) to mark maize SS isozymes in relation to sorghum; lanes 2 and 3, immature endosperm (20 μg) and embryo (20 μg), respectively; lane 4, roots from young seedling (30 μg); lane 5, cell suspension culture (60 μg). Values in parentheses indicate the amount of total soluble protein.

RNA Blots and Anaerobic Expression

The anaerobic induction of the SS genes was examined at the messenger RNA level by Northern blot hybridizations (Fig. 5) using the maize *Sh* and *Sus* cDNA clones and at the protein level by native and denatured Western blot analyses

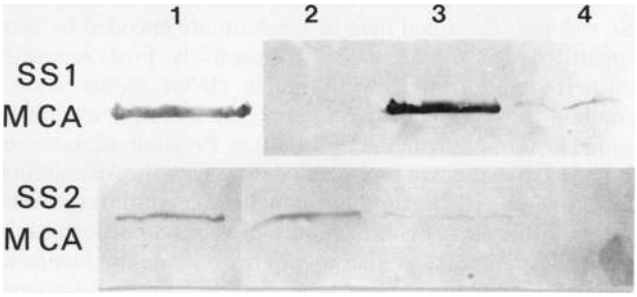

Figure 4. Denaturing Western blot showing sucrose synthase-specific polypeptides in crude extracts from various tissues of sorghum (except lane 1) using maize SS1- or SS2-specific monoclonal antibody, as indicated. Lane 1, developing maize endosperm (2 μg for SS1 and 10 μg for SS2); lanes 2 and 3, developing sorghum embryo (25 μg) and endosperm (25 μg), respectively; lane 4, roots from young seedlings (30 μg). Values in parentheses indicate the amount of soluble protein.

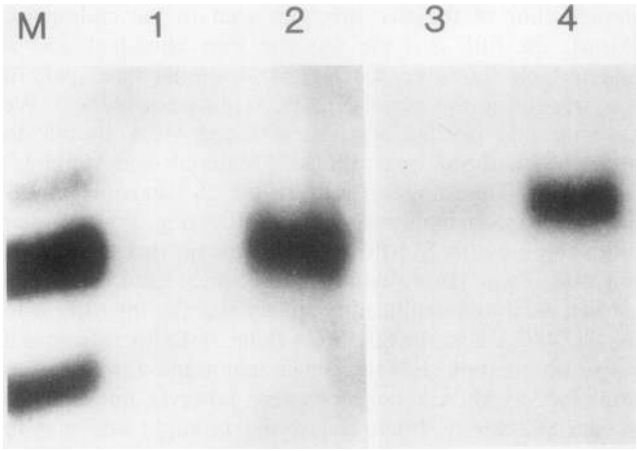

Figure 5. Northern blots showing RNA transcripts corresponding to *Sh* (lanes 1–2) and *Sus* (lanes 3–4) cDNA probes in 0.5 μg poly(A)$^+$ RNA from aerobic (lanes 1 and 3) and anaerobic (lanes 2 and 4) sorghum seedlings. M represents the marker lane.

(Fig. 6). The Northern analysis showed markedly high levels of hybridization signal to both the SS probes in anaerobic samples relative to the control. This indicated that the anaerobic treatment led to a significant increase in the steady-state levels of both the SS transcripts. These analyses also show that, as in maize, the sorghum SS2 transcript was slightly larger than the SS1 transcript and that there was no cross-hybridization with the maize cDNA probes under the stringent conditions of hybridizations and washings. Crude extracts from the same control and anaerobic seedling populations (as the one used for the RNA studies), were analyzed for the SS proteins. Both the control and anaerobic root extracts showed a typical five banded pattern of SS isozymes in native blots (Fig. 6) and a single band in the denaturing blots (Fig. 6). No detectable differences in the intensity of SS bands were seen between the control and anaerobic samples.

DISCUSSION

Various lines of evidence indicate that the two SS isozymes, SS1 and SS2, described here in sorghum are encoded by two nonallelic genes, *Sus1* and *Sus2*, respectively. First, genomic Southern hybridizations with maize cDNA clones corresponding to the nonallelic SS genes, *Sh* and *Sus*, exhibited unique genomic fragments in sorghum. Previous analyses in maize (13) have shown no hybridizations with the *Sh* and *Sus* cDNA probes to their respective nonallelic genomic fragments under the stringent hybridization and wash conditions such as used here. We suggest that the *Sh* hybridizing fragments in sorghum are due to the *Sus1* gene, encoding the SS1 isozyme (see below for more evidence). Similarly, the maize *Sus* cDNA probe detected unique sorghum fragments which we ascribe here as due to the second gene, *Sus2*, encoding the SS2 isozyme. A possibility that the *Sh* and *Sus* specific sorghum fragments are due to allelic genes is ruled out, as it is unlikely that allelic variations will lead to such divergence that no cross-homologies will be detected by the full length cDNA clones of the maize SS genes. Two nonallelic SS genes, based on cDNA sequences and hybridization specificities with the maize *Sh* DNA probe, have also been described in wheat (18). The Northern blot hybridizations (Fig. 5) have similarly detected only the gene-specific transcripts. As in maize, the *Sh* and *Sus* probes have detected 2750 bp and 2900 bp sized transcripts, respectively, in sorghum root RNAs. Because of the size similarities as well as hybridization specificities to the maize clones, we conclude that these two transcripts correspond to *Sus1* and *Sus2* genes. It is noteworthy, however, that the identical sized SS1 and SS2 polypeptides were detected (Fig. 6). A certain portion of the *Sus2* message is possibly not translated, or is translated but does not lead to a detectable change in size of the two polypeptides. A similar situation has been described for the maize *Sus* gene which encodes a larger transcript (13) and an SS2 polypeptide which is detectably smaller in size than the SS1 polypeptide (Fig. 6; 19).

At the protein level, the sorghum immature embryo is characterized by a single major SS band in native and denaturing Western blots. A greater cross-reactivity of the embryo SS protein with the SS2 than the SS1 MCA indicated that it shared greater structural similarity with an epitope unique to the maize SS2 protein. The maize SS2 MCA used here is highly specific as it does not show any cross-reactivity with the maize SS1 isozyme (our unpublished data). We have previously characterized the maize immature embryo isozyme to be SS2-specific (3, 7, 12). Conservations of epitope as well as embryo specificities of the SS2 protein in maize and sorghum are of significant interest. Biochemical studies have shown the SS enzyme to be a tetramer of four identical subunits in a variety of plant species, including mung bean (11), maize (23), and rice (20). Genetic analyses on SS isozymes in maize have confirmed the biochemical data (23) as evidenced by the five SS polymers due to a random copolymerization of SS1 and SS2 subunits (6). In sorghum, the

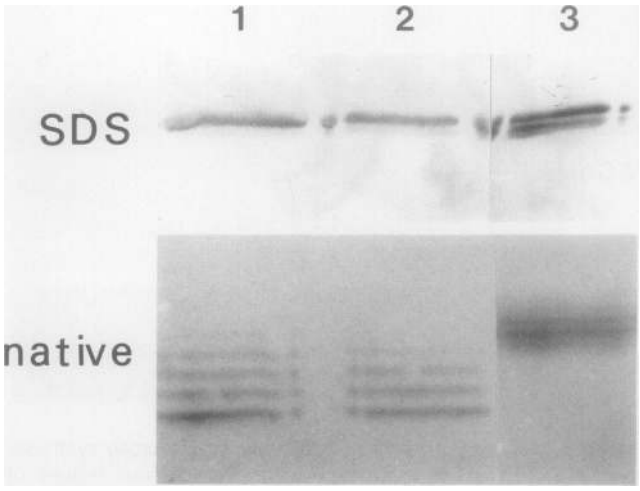

Figure 6. Denaturing (30 μg) and nondenaturing (60 μg) (as indicated) Western blots showing sucrose synthase-specific protein bands, through the maize SS1 polyclonal antibody, in seedling extracts of: lane 1, aerobic control; lane 2, anaerobic; lane 3, maize root extract (30 μg for either type of gel). Values in parentheses represent the amount of soluble protein.

presence of five SS isozymes and the subunit size as identical to the maize SS subunits, are strongly suggestive that the sorghum SS enzyme is a tetramer of four identical subunits. As in maize, the five isozymes are attributable to a random copolymerization of SS1 and SS2 subunits leading to two homotetramers, S1S1S1S1 and S2S2S2S2 and three heterotetramers, S1S1S1S2, S1S1S2S2, and S1S2S2S2 in endosperm and root cells of sorghum. Assuming that the subunits copolymerize randomly, the greater or the lesser intensities of the bands at the slower or faster migrating end of the cluster of the bands are related to the relative *in vivo* abundance of SS2 or SS1 subunits, respectively. The lack of cross-reactivity between the SS2 monoclonal antibody and the heteromeric isozymes suggested that the epitopic residues were not as accessible as with the homotetramers, presumably due to conformational changes in the hybrid protein. In this regard, the SS1 monoclonal antibody reacted differently as both homomeric and heteromeric isozymes were cross-reactive.

The *Sus1* gene in sorghum endosperm was far more abundantly expressed than the *Sus2* gene as judged by the intensities of SS protein bands in native (Figs. 2 and 3) as well as in denaturing gels (Fig. 4). A major difference between sorghum and maize, in terms of expression of the SS genes in endosperm, was related to the presence or the absence of the SS heteromers. The lack of SS heteromers in maize is predominantly attributable to spatial separation in the expression of the two SS genes (1, 14). Immunohistological analyses show that aleurone and the basal transfer cells are specific to the SS2 protein, and the storage cells are characterized by the SS1 protein. The spatial differences in the expression of the two genes in maize are reflective of their differential *in vivo* functions and also, possibly, tissue-specific intergenic regulatory circuits, allowing the expression of only one of the two genes, particularly in endosperm/embryo cells (1, 14). Although similar immunohistological studies in sorghum remain to be done, the presence of SS heteromers suggests that both the SS genes were simultaneously expressed, at least in some of the cells in the sorghum endosperm. Despite these differences, the tissue-specific signals which control greater abundance of the SS1 protein in the endosperm are conserved between maize and sorghum. The anaerobic induction clearly led to an increase in the steady-state levels of both the SS RNAs in sorghum; however, no concomitant change in the levels of the SS proteins was seen. In maize, a similar induction is limited to the *Sh* locus (19, 22), and the posttranscriptional blockage appears to be operative beyond the polyribosomal loading of the message (24). The only exception is the meristematic nondifferentiated root tip region which exhibited a slight elevation of the SS1 protein, as judged by the *in situ* cellular level studies on tissue sections of maize (21). Thus, although both crop plants show much posttranscriptional regulation, both the SS genes were inducible in sorghum and only one of the two genes is anaerobically responsive in maize.

In conclusion, the data presented here show that the two genes, *Sh* and *Sus*, in maize (12, 13) are more similar to *Sus1* and *Sus2* genes in sorghum, respectively, than to each other. The structural similarities relating to regulatory features which determine endosperm/embryo specificities and the posttranscriptional regulations of the anaerobic induction were also conserved. We suggest that gene duplication arose prior to the evolutionary separation of these two plant species. The subtle difference in the anaerobic induction of the *Sus2* gene in sorghum relative to the *Sus* gene in maize is, however, intriguing. Another difference of much interest is the presence of heterotetramers in sorghum endosperm which indicates that, unlike maize where only the *Sh* or the *Sus* is expressed, both the SS genes were expressed simultaneously. It is possible that these minor variations arose subsequent to the evolutionary divergence of maize and sorghum.

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