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Spatial and temporal expression of the two sucrose synthase genes in maize: immunohistological evidence

Y.-C. Chen [1] and P.S. Chourey [1,2,*]

[1] Department of Agronomy, University of Florida, Gainesville, FL 32611, USA
[2] Department of Plant Pathology and US Department of Agriculture/Agriculture Research Service, University of Florida, Gainesville, FL 32611, USA

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Summary. We describe the spatial and temporal immunohistological distributions of the two sucrose synthases, SS1 and SS2, encoded by the *Sh* and *Sus* genes, respectively, in different parts of the maize plant. The two similar isozymes were differentially localized in developing endosperm cells through the combined uses of a *shrunken* (*sh*) mutant lacking the SS1 protein and the SS1 and SS2 antisera. The accumulation of SS1 protein always coincided with starch deposition in the *Sh* endosperm cells, whereas in the *sh* endosperm, the centrally located cells were lost at or during the most critical phase of starch biosynthesis. The SS2 specific cells, including aleurone layer and the basal endosperm transfer cells in both genotypes, were not associated with detectable starch deposition. Such heterogeneity was indicative of two cell types separable by gene expression, and of differential in vivo roles of the two isozymes in the endosperm. In young roots, the expression of both SS encoding genes was predominantly in the vascular cylinder region. These data fulfill a previous prediction, based on the genetic analyses, that the expression of the SS genes is spatially and/or temporally separated in endosperm cells but not in root cells.

Key words: Kernel – Seedling – Starch biosynthesis – Cell specific – Gene expression

Introduction

The two sucrose synthase genes in maize constitute an ideal system to analyze gene expression at the cellular level in relation to development/differentiation, and to elucidate functional roles of the encoded isozymes in the plant. Of the two genes, the *shrunken* (*Sh*) gene encodes sucrose synthase-1 (SS1) protein, which constitutes a critical link in starch biosynthesis in developing endosperm (Chourey and Nelson 1976). Complete absence of the SS1 protein, as in the *sh* mutant, is associated with a slight starch-deficient endosperm phenotype. In addition, an extensive analysis of many carbohydrate enzymes at various developmental stages of maize kernel has shown a positive correlation between the levels of SS activities and starch content (Tsai et al. 1970). Specifically, a sharp rise in SS activity (and several other enzymes) at 12 days after pollination (DAP) is associated with the onset of active starch accumulation starting at about 14 DAP stage. No other plant part is phenotypically affected by the *sh* mutation. The *Sh* expression is marked by an endosperm-specific abundance, while there is a lack of any detectable level in the embryo of the immature kernel (Chourey and Nelson 1976; Schwartz 1960; Chourey 1981; Echt and Chourey 1985).

The second sucrose synthase, SS2, is encoded by the *Sus* gene (Chourey 1981, designated previously as *Ss2*). The *Sus* gene is expressed in many tissues, including embryo; however, it does not exhibit endosperm-specific abundance (Echt and Chourey 1985; Chourey 1981; Chourey et al. 1988). Recently, the first *Sus*-null mutation has been described and characterized by the lack of SS2 protein in all tissues analyzed; interestingly, no detectable phenotypic change is seen with the loss of this protein (Chourey et al. 1988). At the molecular level, both genes have been cloned and extensively characterized (Werr et al. 1985; McCarty et al. 1986; Gupta et al. 1988). The *Sh* and *Sus* loci are mapped to the short and long arms of chromosome 9, respectively; the latter is about 40 map units from the former (McCarty et al. 1986; Gupta et al. 1988).

* To whom correspondence should be addressed

The enzyme sucrose synthase (EC 2.4.1.13) catalyzes the following reversible reaction:

Sucrose + UDP↔UDP glucose + Fructose.

Because sucrose is the major transport sugar, including the long distance source to sink transport, this enzyme, along with sucrose P synthase and invertase, plays a key role in sucrose metabolism in plants. Extensive biochemical and physiological studies on this enzyme have been reported (Giaquinta 1980). Briefly, the enzyme plays a major role in sucrose→starch conversion reactions. The UDP-glucose can serve as a precursor for polysaccharide synthesis or can be converted to glucose-6-P through the relevant enzymes and enter the respiratory pathway. The tetrameric structure of the maize sucrose synthase has been demonstrated first by biochemical (Su and Preis 1978) and later by genetic analyses (Chourey et al. 1986). In maize, the two isozymes, SS1 and SS2, are extremely similar in their biochemical properties (Echt and Chourey 1985; Chourey 1981). The major difference, as indicated before, is in their tissue distributions in the plant. Another difference appears to be in their inductional properties. Anaerobic induction of the *Sh* but not the *Sus* gene (Springer et al. 1985, 1986; McCarty et al. 1986) indicates a possible role of the SS1 protein in anaerobic glycolysis. Although such a possibility is not entirely ruled out, the high level of anaerobic induction of the *Sh* transcripts is, however, not associated with a detectable increase in the SS1 protein levle (McElfresh and Chourey 1988). This aspect raises some interesting questions regarding post-transcriptional regulation of the *Sh* gene.

Another observation of much interest is related to the tissue-specific polymerization of the SS1 and SS2 subunits (Chourey et al. 1986). Immuno-blot analyses of the *Sh* genotype have shown five SS isozymes comprising two homotetramers and three heterotetramers in root extracts, whereas only the two homotetramers are seen in endosperm extracts. The lack of heteromers has been attributed to a spatial/temporal separation in the expression of the two genes in endosperm cells (Chourey et al. 1986). This observation is significant both in terms of physiological roles of the two isozymes in endosperm as well as the in terms of the developmental biology of the endosperm tissue itself. Kiesselbach (1949) provided the most detailed histological description of the developing maize kernels. Anatomical and cytological heterogeneity in developing endosperm has also been reported (McClintock 1978). Molecular heterogeneity in terms of differential DNA content per nucleus in endosperm cells is also recently described (Kowles and Phillips 1985). The immuno-histological data presented here demonstrate spatial heterogeneity in *Sh* and *Sus* expression in developing endosperm cells. In addition, we suggest that the differential localization of the two isozymes is indicative of their different in vivo functions in various parts of the plant.

Materials and methods

Immature kernels at 12 and 16 days after pollination (DAP) and roots from 5- to 7-day-old seedlings of *Sh Sh* or *sh*-deletion stock (*sh bz-m4*) were fixed in formalin acetic acid, dehydrated through a tertiary butyl alcohol series and cast into paraffin blocks as described (Sass 1945; Clark and Sheridan 1986). Preparation of mono-specific SS1 and SS2 antisera has been described previously (Echt and Chourey 1985). The immuno-gold silver stain (IGSS) reactions on 10 μm thick sections were done according to the manufacturers (Janssen Life Products) specifications. Briefly, deparaffinized slides, subsequent to rehydration and blocking with 0.5% BSA-TRIS plus 20 m*M* NaN3 solution, were treated with 50–200 μl of 5% normal goat serum (NS). After 20 min, NS was replaced with an equal volume of 1:500 diluted SS1 and 1:200 diluted SS2 antibodies. These levels of dilutions were determined to be the highest dilutions which resulted in a positive signal for the respective proteins. The overnight treatment with primary antibodies was followed by three washes with 0.1% BSA-TRIS + 20 m*M* NaN3 solution. The slides were then treated with 100-fold diluted secondary antibody comprised of goat anti-rabbit IgG linked to colloidal gold (IGS) for 30 min. After washing off the IGS solution, post-fixation of the preparation was done using 2% glutaraldehyde for 15 min. After two washes with PBS and three washes with water (2–3 min each), the slides were washed with 0.2 *M* citrate buffer for 2 min. The final step included the incubation of slides for 7 min in freshly prepared silver-enhancement reagents. Slides were then made permanent after dehydration through an ethanol series (30%–50%–70%–85%–95%–100%–xylene). Staining with Fast Green was done at the 95% ethanol level. Qualitative judgements on approximate cellular levels of SS proteins were based on the level of color intensities on tissue sections treated with SS1 or SS2 antibody, as compared to the pre-immune sera. Darker precipitates indicated more SS cross-reactive material than the faintly stained cells.

Results

Serial sections of immature kernels and young roots of 5- to 7-day-old seedlings of *Sh* and *sh* genotype were examined for the accumulation of SS1 and SS2 isozymes encoded by the sucrose synthase genes. Several developmental stages of the kernel were examined; 12 ad 16 DAP stages were of particular interest, as the *Sh* locus is known to undergo a major increase in expression during this specific phase of development (Chourey 1981). Immuno-localization was done using either SS1 or SS2 antibody followed by a secondary antibody conjugated to immuno-gold. Kernel sections were also stained with I-KI for starch localization.

Figures 1 and 2 present photographs showing cellular localizations of SS proteins and starch in immature endosperm and embryo at 12 and 16 DAP stages, respectively, in the *Sh* and *sh* genotypes. The lack of IGSS with a pre-immune serum and a strong signal with the SS1

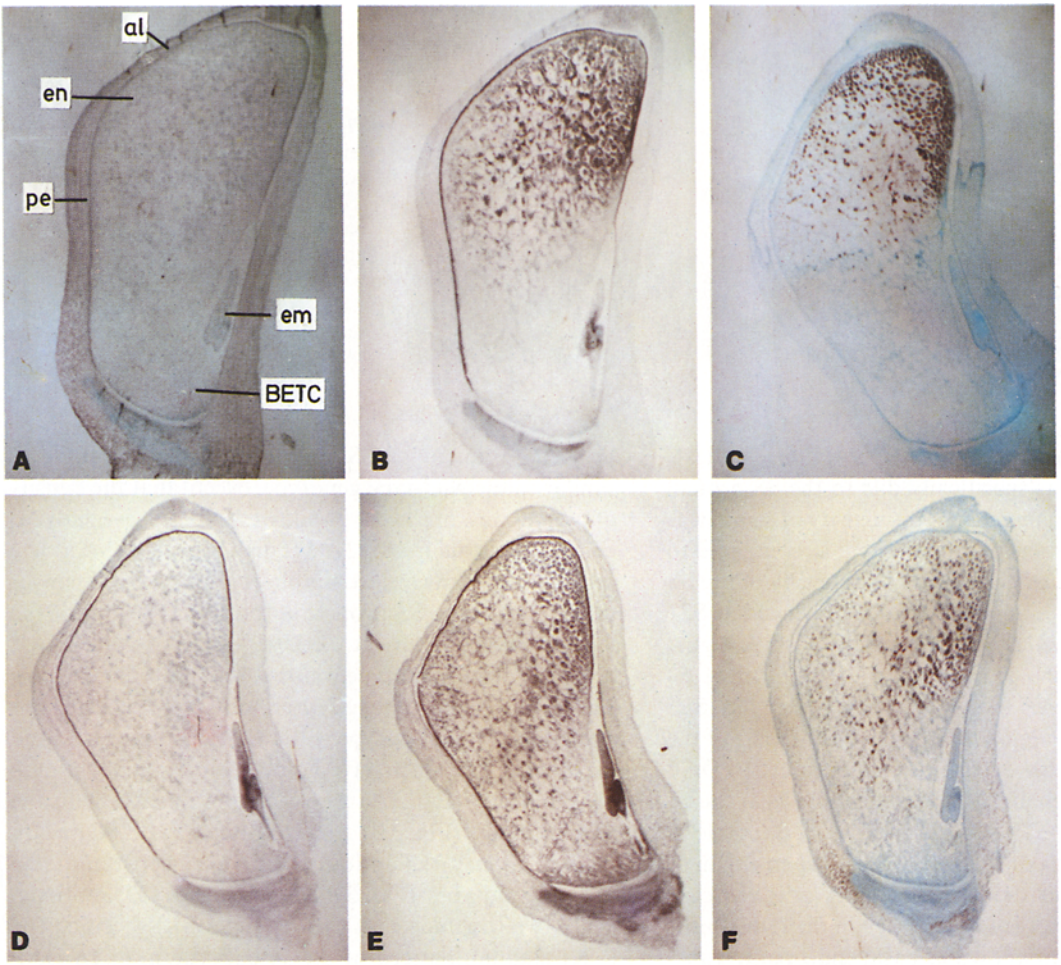

Fig. 1 A–F. Localizations of SS proteins and starch in 12 DAP kernels of *Sh* and *sh* genotypes. Longitudinal section of paraffin-embedded *Sh* (**A–C**) and *sh* (**D–F**) kernels at 12 DAP stained with Fast Green plus as indicated; **A** – pre-immune serum, **B** – SS1 antiserum, **C** – I-KI, **D** – SS1 antiserum, **E** – SS2 antiserum and **F** – I-KI. Abbreviations are: *al* – aleurone; *en* – endosperm; *em* – embryo; *pe* – pericarp; (original maginfication × 7)

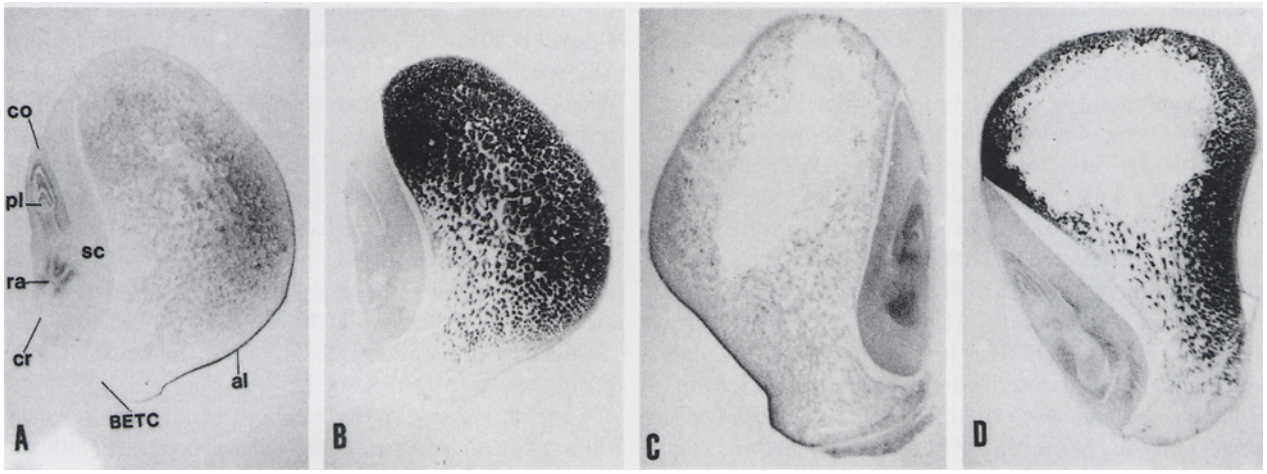

Fig. 2 A–D. Localizations of SS proteins and starch in 16 DAP kernels of *Sh* and *sh* genotypes. Longitudinal section of paraffin-embedded *Sh* (**A–B**) and *sh* (**C–D**) kernels at 16 DAP stained with Fast Green plus as indicated; **A** – SS1 antiserum, **B** – I-KI, **C** – SS2 antiserum and **D** – I-KI. Abbreviations are; *al* – aleurone; *co* – coleoptile; *pl* – plumule; *sc* – scutellum; *ra* – radicle; *cr* – coleorhiza; (original magnification × 7)

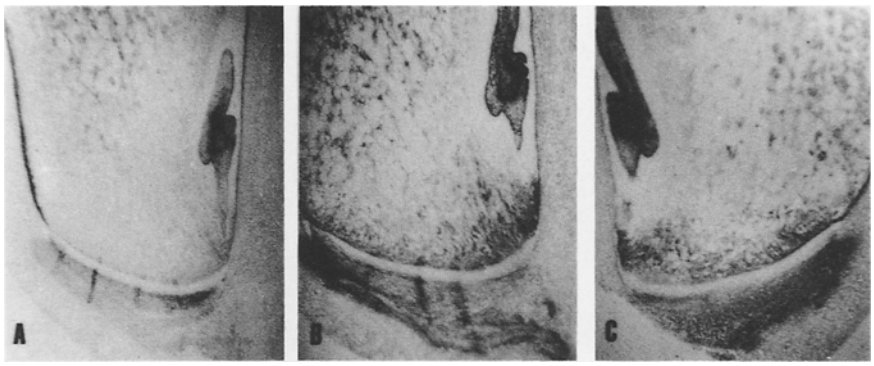

Fig. 3A–C. Localizations of SS proteins in the basal region of the kernel. A close-up view of the longitudinal section of the kernel showing the basal region at the 12 DAP: *Sh* (**A** and **B**) and *sh* (**C**) genotypes stained with Fast Green plus as indicated; **A** – SS1 antiserum, **B** and **C** – SS2 antiserum; (original magnification ×18 (**A**) and ×20 (**B** and **C**)

antibody in *Sh* endosperm cells at 12 DAP stage indicated that the signal was specifically due to the SS protein (Fig. 1). A significant increase in the density of signal was seen at the 16 DAP stage of development as compared to the early stages (12 DAP). Because nearly 95% of the SS protein in *Sh* endosperm is due to the *Sh*-encoded SS1 protein (Chourey 1981), we believe the positive signal seen here is due to the *Sh* expression. A gradient of *Sh* expression at 12 DAP, i.e. much higher level of SS1 protein in the crown region than in the central part of the endosperm, was readily detectable (Fig. 1B). At 16 DAP the spatial pattern remained nearly the same as at the 12 DAP stage (Fig. 2A). The SS1 protein was detectable in a much larger portion of the 16 DAP endosperm than at the 12 DAP stage of development. It is of significant interest that no *Sh* expression was seen in the lower-most part of the endosperm known as basal endosperm transfer cells (BETC) (Figs. 1B and 2A). The staining pattern for starch at 12 and 16 DAP coincided with the *Sh* expression in the *Sh* endosperm (Figs. 1B–C and 2A–B). No starch accumulation was seen in the BETC region (Figs. 2B and D).

Similar analyses were done on the *sh*-deletion strain, *sh bz-m4*, thus enabling a specific localization of only the SS2 protein in this genotype. The SS1 and SS2 proteins are immunologically similar but not identical (Echt and Chourey 1985); Western blot analyses show a stronger immuno-signal in a homologous antigen-antibody reaction (i.e. SS1 antigen with the SS1 antibody) than a heterologous reaction (data not shown). As expected, the IGSS reaction with the SS2 antibody (Fig. 1E) was much stronger than with the SS1 (Fig. 1D) at the 12 DAP. At 16 DAP, cells in the central region of the *sh* endosperm were lost (Fig. 2C–D). This is a significant observation which has not been described previously. Kernels at the 14 DAP stage were also examined; only a column of cells connecting the crown cells with the basal cells was seen and the cells on each side of this column were lost (data not shown). A complete loss of the central part of the endosperm provides the causal basis for the *shrunken* phenotype subsequent to desiccation in the mature kernel. The gradient of SS2 accumulation was otherwise similar to the wild-type genotype, except that the density of signal was markedly weak at both stages analyzed (Figs. 1B versus E and 2A versus C).

Figures 3 shows close-up photographs of cellular localizations of the SS protein in the BETC region of the *Sh* and *sh* genotypes at 12 DAP stage. An important feature of the *sh* genotype was that the BETC region gave a positive signal with the SS2 antibody (Fig. 3). An examination of the *Sh* genotype with the SS2 antibody showed that the BETC which gave no detectable signal with the SS1 antibody (Fig. 3A) were actually positive with the SS2 antibody (Fig. 3B). In addition, the BETC region showed no major quantitative difference between the *Sh* and *sh* genotypes (Fig. 3B–C) with the SS2 antibody, hence, we conclude that this region is SS2-specific. The I–KI staining pattern of the *sh* endosperm at 16 DAP was similar to the *Sh* endosperm, except for the empty area in the central part of the endosperm (Fig. 2D). As in the *Sh* genotype, no starch deposition was detected in the BETC in the mutant genotype (Figs. 1F and 2D). The most intense signal for the SS proteins was seen in the cells in the aleurone layers of both genotypes (Figs. 1B, D and E). We believe the aleurone layer is also SS2-specific, as no major differene in signal density was detectable between the two genotypes. A sharp discontinuation in the immuno-signal was seen at a point where these cells develop into the BETC region. This is readily detectable in sections reacted with the SS1 antiserum, as the SS2-specific basal cells were unreactive and thus yielded a better contrast for such a delineation (Figs. 1D and 3A). A greater density of immuno-signal in the aleurone layer was seen at 12 DAP (Fig. 1B, D and E); a reduced signal was detected in the crown region of both genotypes at 16 DAP (Fig. 2A and C).

The developing embryo in both genotypes showed a similar pattern of SS immuno-localization. A significant level of differentiation at 16 DAP was correlated with a high level of tissue and cellular specificity in SS expression. For example, we detected a much lower signal density in the scutellar region than in coleoptile, primordial

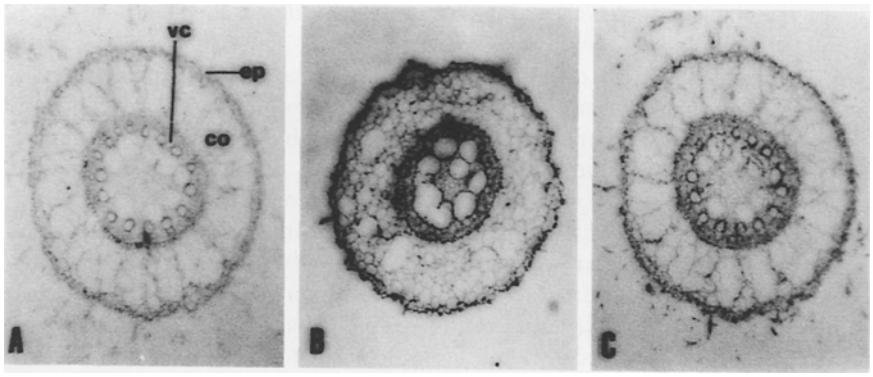

Fig. 4 A–C. Localization of SS proteins in young roots of *Sh* and *sh* genotypes. Transverse section of roots of *Sh* (**B**) and *sh* (**A, C**) genotypes stained with Fast Green plus as indicated; **A** – pre-immune serum; **B** and **C** – SS1 antiserum. Abbreviations are: *ep* – epidermis; *co* – cortex; *vc* – vascular cylinder; (orginal magnification ×25)

leaves, radicle and coleorhiza (Fig. 2 A and C). Previous data have indicated that there is no *Sh* expression in immature embryo, as only the SS2 protein was seen in the embryo extracts (Chourey and Nelson 1976; Schwartz 1960; Chourey 1981; Echt and Chourey 1985). A slightly higher signal density with the SS2 antiserum in the *sh* kernel (Fig. 2 C) than with the SS1 antiserum in the *Sh* embryo (Fig. 2 A) substantiates the earlier observations.

The pedicel region at the base of the *Sh* kernel reacted faintly with the SS1 antibody but yielded a strong signal with the SS2 antibody (Fig. 3 A and B, respectively). No major detectable difference in signal density was seen between *Sh* and *sh* pedicels with the SS2 antiserum (Fig. 3 B and C, respectively). Figure 4 presents a photograph showing cellular localizations of SS proteins in young roots. Transverse sections of *Sh* and *sh* roots of 5- to 7-day-old seedlings were also examined immuno-histologically (Fig. 4). A much greater IGSS signal was seen in the vascular cylinder and epidermal cells than in the cortex. No cell-specific difference was detected between the two genotypes using either the SS1 or the SS2 antibody.

Discussion

Immuno-histological data presented here demonstrate spatial heterogeneity in gene expression in developing endosperm cells of maize. The two very similar sucrose synthase isozymes, SS1 and SS2 (Echt and Chourey 1985; Chourey 1981), encoded by the *Sh* and the *Sus* loci, respectively, were differentially located in several parts of the endosperm. We suggest that this heterogeneity is indicative of different functions of the two isozymes in sucrose metabolism in this important tissue of the maize plant. The physiological role of the *Sh*-encoded SS1 isozyme in mobilizing photosynthesized sucrose into starch biosynthesis has been demonstrated previously through genetic analyses (Chourey and Nelson 1976, 1979). Further support of such a role of the SS1 isozyme was evident in the spatial and temporal correlation seen here between the in situ localization of the SS1 protein and starch-accumulating cells in the *Sh* endosperm. As starch and the SS1 protein are not transportable intercellularly, the coincidences of I–KI and IGSS signals with SS1 antibody identify individual cells active in the sucrose → starch conversion reactions. Enzymatic analyses on complementing heterozygotes relative to the *sh* homozygous parents have previously indicated that sucrose cleavage role might be the critical physiological function of the SS1 protein (Chourey and Nelson 1979).

The SS2-specificity of the BETC and the aleurone layer were demonstrated through the *sh* mutation lacking the SS1 protein and by using the SS2 antibody. The BETC are modified in structure and are characterized by immense surface of wall in-growths, as observed by transmission electron microscopy (Shannon et al. 1986). We describe here several additional distinctive features of these cells, which include the specific localization of the SS2 protein, lack of the SS1 protein and no detectable deposition of starch. We suggest that the SS2 protein in these conducting cells might be involved in symplastic resynthesis of sucrose from the monosaccharides inverted from sucrose at the base of the kernel. Pulse labelling experiments have demonstrated (Shannon 1972) that labeled glucose and fructose predominate in the lower portion of the endosperm, and the proportion of labeled sucrose becomes greater in the upper parts of the endosperm active in starch synthesis. It is noteworthy that starch accumulation in the *sh* endosperm in the crown region and below the cavity area coincided with low levels of SS2 protein in these cells. Whether the SS2 protein in these starch-accumulating cells played the same role as the SS1 protein in the *Sh* genotype (i.e. sucrose cleavage) remains unclear. A similar functional role for the SS2 protein will need to be invoked for the scutellar cells of 16 DAP embryo, which also showed a coincidence of SS2 protein and starch deposition. Physiological significance of SS2 protein accumulation in the pedicel region at the base of the kernel is unclear.

It is noteworthy that the SS2-specific cells, unlike the cells showing the SS1 isozyme, were often not associated

with starch deposition. Such cell types and tissues included aleurone layer, BETC, pedicel region and the plumule-radicle axis in the developing embryo. The temporal changes in the aleurone layer as evidenced by the gradual extinction of the SS2 signal with the advanced maturity of the kernel are most likely related to the presently unknown SS2 function(s) in aleurone cells. The triploid cells of the aleurone layer are well-marked for anthocyanin pigment; they also harbour protein granules, catalase-2 isozyme (Tsaftaris and Scandalios 1986) and lipid storing sphorosomes (Kyle and Styles 1977). Similarly, the young differentiating proembryo undergoes numerous complex metabolic reactions. The SS2 isozyme may mobilize sucrose into divergent pathways in such metabolically active cells. The recent isolation of the first *Sus* mutant showing no detectable SS2 protein in all tissues of the plant (Chourey et al. 1988) would be of special interest for such in situ localization studies.

The *sh* kernel during the period between 12 and 16 DAP stages showed specific loss of the central part of the endosperm. This period is critical for the centrally located cells in the endosperm for the occurrence of numerous developmental events. The most important changes include cell expansion (Kiesselbach 1949), the initiation of probably the most rapid phase of starch deposition (Tsai et al. 1970) and marked increase in nuclear size and DNA content per nucleus (Kowles and Phillips 1985). Because the central endosperm cells in the *sh* genotype begin to breakdown just prior to or during these major alterations, the role of the SS1 protein in endosperm development assumes significant importance. We suggest that perhaps the *sh* cells cannot withstand the stress of cell expansion and/or that translocated sucrose from BETC region leads to an osmotic imbalance relating to the lesion in the sucrose to starch reactions. It is worth pointing out, in the context of the latter hypothesis, that endosperm cell walls at 12 DAP stage are known to be exceptionally thin and fragile (Phillips et al. 1985). It is unlikely that the loss of these cells is related to their starch deficiency, as the similar trait of the BETCs did not lead to the degradation in this region of the endosperm.

Finally, these data also provide an important insight into a previously described tissue-specific polymerization of the SS1 and SS2 subunits in the *Sh* genotype (Chourey et al. 1986). The presence of the two homotetramers, S1S1S1S1 and S2S2S2S2, and the lack of the three heterotetramers, S1S1S1S2, S1S1S2S2 and S1S2S2S2 led us to suggest, among other possibilities, that the expression of the *Sh* and *Sus* genes is spatially separated in the endosperm cells. Indeed, the data presented here indicate a significant level of spatial distinction in the expression of the two genes in the endosperm. However, a low level of the SS2 protein was detected in the central part of the *sh* endosperm, the same region which exhibited abundant levels of the SS1 protein in the *Sh* endosperm at the 12 DAP stage. Whether both the genes are expressed in this part of the *Sh* endosperm remains to be demonstrated. It is possible that if both gene products are targeted for the same metabolic pathway (such as sucrose → starch reactions) that a preponderence of one, such as the SS1 protein, might turn off the second gene. This may explain why the SS heterotetramers are not detectable in the *Sh* endosperm (Chourey et al. 1986). Alternatively, extremely low levels of the SS2 subunits, relative to the abundant SS1 levels, in this region of the *Sh* endosperm may yield undetectable levels of such heterotetramers. Localization of SS proteins in root cells follows the previously predicted pattern on the basis of genetic analyses (Chourey et al. 1986). Both SS1 and SS2 proteins were localized mainly in the vascular cylinder region and in the epidermal cells; very low levels of signal were evident in the cortical cells. Because there was no cell-specific immuno-histological difference between the two genotypes and because of the previous demonstration of SS heterotetramers in root extracts, we conclude that both genes are expressed simultaneously in the same cells.

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