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**The Importance of the Enzyme Sucrose Synthase
for Cell Wall Synthesis in Plants**

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The Importance of the Enzyme Sucrose Synthase for Cell Wall Synthesis in Plants

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Figures of unpublished data plus publications resulting from this grant are attached at end of this report.

I. Abstract

The goal of this work was to understand the role of the enzyme sucrose synthase (SuSy) in synthesis of cellulose and callose in plants. The work resulting from this grant leads to a number of conclusions. SuSy clearly plays diverse roles in carbon metabolism. It can associate with the plasma membrane of cells undergoing rapid cellulose deposition, such as cotton fibers, developing maize endosperm, gravistimulated pulvini, and transfer cells of the cotton seed. It is also concentrated at sites of high callose deposition (tapetal cells; cell plates). When SuSy levels are lowered by mutation or by anti-sense technology, cell walls undergo degeneration (maize endosperm) and show reduced levels of cellulose (potato tubers). In sum, our evidence has very much strengthened the concept that SuSy does function in the plasma membrane to channel carbon from sucrose via UDP-glucose to glucan synthase complexes. Soluble SuSy also clearly plays a role in providing carbon for starch synthesis and respiration. Surprisingly, we found that the cotton seed is one unique case where SuSy apparently does not play a role in starch synthesis. Current evidence in sum suggests that no specific SuSy gene encodes the membrane-associated form, although in maize the SS 1 form of SuSy may be most important for cell wall synthesis in the early stages of endosperm development. Work is still in progress to determine what does control membrane localization—and the current evidence we have favors a role for Ca^{2+} , and possibly also protein phosphorylation by differentially regulated protein kinases. Finally, we have discovered for the first time, a major new family of genes that encode the catalytic subunit of the cellulose synthase of plants—a result that has been widely cited and opens many new approaches for the study of this important plant function.

II. Objectives of the Original Research Proposal

The overall objective this research was to provide a clearer understanding of the physiological roles of the enzyme sucrose synthase (SuSy) in higher plants. SuSy catalyzes the following reversible reaction: $\text{sucrose} + \text{UDP} \leftrightarrow \text{UDP-glc} + \text{fructose}$. This enzyme, always considered to be localized in the cytoplasm, has for many years been recognized as being very important in the cleavage of sucrose for use in starch synthesis in non-photosynthetic tissues. Work from our laboratories had recently provided data which indicated that SuSy may play an additional and critical role in provision of nucleotide sugar precursor UDP-glc for the biosynthesis of the β -glucans callose and cellulose. Those discoveries, that led to this proposal, were: 1) that developing endosperm cells of maize which essentially lack SuSy activity due to a mutation at the *Sh* locus that encodes the endosperm-specific form of SuSy, are also brittle and undergo early cell wall degeneration indicative of altered patterns of wall synthesis (Chourey and Miller, 1991); 2) the discovery of a substantial level of a membrane-associated form of sucrose synthase in developing cotton fibers (a non-starch producing cell type) at the time of active secondary wall cellulose synthesis (unpublished at that time). We thus proposed to define more precisely the role of sucrose synthase in cell wall synthesis in maize and cotton.

Our original specific objectives included: a) to attempt to determine whether the cell walls of mutant *sh1* endosperm to see if there was a difference in the pattern of polysaccharide deposition in mutants lacking SuSy activity; b) to study the distribution of soluble vs. membrane-bound isozyme forms of SuSy in various cell types of maize and

cotton; d) to understand the requirements for membrane localization of SuSy and to study the regulation of this form with respect to its potential role in a coupled reaction in which sucrose is ultimately converted to cellulose and/or callose; e) to clone and study the expression of SuSy genes in cotton in relation to cell wall synthesis.

As for most projects, some of these goals were completed as anticipated, and some proved more difficult than expected. In addition, some new and unexpected findings also led us in some directions different than we had originally predicted.

III. Relevant data, methodology, results, and discussion

(note: publications resulting from this grant are highlighted in bold in the references cited in the text below)

a) Membrane-Associated SuSy in Cotton and Maize.

In the early stages of this grant proposal, work was continued to clarify the discovery of membrane-associated SuSy in developing cotton fibers. This work then resulted in a publication (**Amor et al., 1995; see attached reprint for detailed description of this work**). In sum, this work clearly showed that a high percentage of the total SuSy of cotton fibers is found associated with the membrane fraction. By using detached and permeabilized cotton fibers, we showed that carbon from sucrose can be converted at high rates to both cellulose and callose. Callose synthesis was favored by inclusion of Ca^{2+} in the buffers, a condition known to favor callose synthase, whereas cellulose synthesis was favored when Ca^{2+} was not present. These results are in marked contrast to the situation when UDP-glc is used as substrate; in this case, only callose is produced suggesting that the coupled reaction involving SuSy can be more favorable for cellulose synthesis. However, it should be noted that the rates observed with this coupled reaction

are extremely variable, and we are still struggling to understand what conditions are needed to stabilize the system to consistently obtain high rates of glucan synthesis from sucrose *in vitro*. In addition, immunolocalization studies reported in this paper show that SuSy can be localized at the fiber surface in helical arrays that exactly parallel the pattern of cellulose deposition, providing further support for the notion that SuSy and cellulose synthase form a complex at the plasma membrane.

More recent unpublished work from DD's lab has confirmed that SuSy is indeed found in the plasma-membrane enriched fraction of cotton (total membranes were used in the previous work). Significantly, we have recently shown that , in extracts prepared in the presence of low levels of Ca^{2+} , a high proportion of the M-SuSy can be eluted from purified plasma membranes or crude high-speed membranes by washing them with EGTA (unpublished, see Figure 1). This suggests that Ca^{2+} may play some role in membrane localization. A very surprising result came when extracts were initially prepared in the presence of EGTA, a condition where very little SuSy remains in the plasma membrane and the soluble fraction contains now a mixture of what was *in vivo* both S- and M-SuSy (See Figure 2). If Ca^{2+} is added back to the soluble fraction to the level of about 1 mM, a precipitate forms that contains a certain proportion of the total SuSy. We presume that the proportion that can be precipitated comes from that which was originally M-SuSy, since no SuSy precipitates from the soluble fraction if extracts were prepared in the presence of Ca^{2+} where M-SuSy is still attached to the membranes (compare Figures 1 and 2). This Ca^{2+} -precipitate is a curious mixture of specific proteins that contain, in addition to M-SuSy, also tubulin, annexins, 14-3-3 proteins, and a small amount of the small G-protein Rac13. Note that the levels of SuSy, tubulin and

Rac13 are much more abundant at the stage of secondary wall synthesis (17 and 21 days post-anthesis) compared to the stage of primary wall synthesis (10 days post-anthesis). We believe these results are trying to tell us something important about possible interactions of M-SuSy with other membrane proteins, and are working now vigorously to try to understand this phenomenon.

Another work carried out in PC's lab has shown that there are high levels of SuSy in tapetal cells of maize—a site of high callose deposition (**Chourey and Miller, 1995; see attached manuscript for details**). The work of Amor et al. (1995) also suggested that SuSy might channel carbon to callose synthase complexes as well as cellulose synthase complexes; this is because sucrose served as a good substrate in vitro for both cellulose and callose synthesis, and also because we occasionally saw SuSy localized in fibers in a punctate pattern that is very similar to the pattern of callose deposition in wounded fibers. In addition, Haigler's group (with whom we collaborated to do the immunolocalizations) has also observed SuSy localized both at sites of high cellulose deposition and at the cell plate of dividing *Zinnia* mesophyll cells, a site of callose deposition (C. Haigler, personal communication).

PC's laboratory has carried out a detailed analysis of SuSy distribution in maize (**Carlson and Chourey, 1996; see attached manuscript for details**). In sum, these studies showed that both isozymes SS1 and SS2, encoded by the loci *Sh1* and *Sus1* respectively, were detected in the plasma membrane-enriched fraction of maize endosperm. In addition, measurement of SuSy activity showed high levels of specific activity for the enzyme in this fraction. The enzyme is tightly associated with the membrane. It is noteworthy that both forms of the enzyme were detectable as both

soluble and plasma-membrane associated forms. The percentage in the membrane was considerably lower than we have observed in cotton fibers that synthesize huge amounts of cellulose, but notably, it was higher in the endosperm during the phase of cell expansion when cell wall synthesis is most active.

b) Cell wall composition in cells possessing mutant SuSy

One of our first goals was attempt to determine whether cell wall composition, in particular the level of cellulose, might be reduced in the endosperm cells of the *sh1* mutant of maize. This turned out to be much more technically difficult that we thought, and, although much effort was invested, in the end we were not able to isolate sufficiently pure endosperm cell walls to perform acceptable analyses. The endosperm tissue is a tiny enough proportion of the seed, that we found that even minor contamination with other vascular tissues and starchy endosperm confounded our analyses. Prem Chourey's group (PC) did the work on cell wall isolation, and while Deborah Delmer's group (DD) did the carbohydrate analyses. No publication therefore resulted from these efforts.

DD's group then took an alternate approach and obtained lyophilized young tubers from potatoes that had been engineered by expression of an anti-sense construct of the SuSy gene to express much reduced levels in SuSy in the developing tubers (Zrenner et al., 1995). Dr. Uwe Sonnewald kindly provided these tissues. Cell walls were prepared, and cell wall compositions were determined by DD's group. As shown in the attached Figure 3, the starch content of tubers of three different transgenic anti-sense SuSy lines T-99, T-129, and T-112 is reduced to different extents with line T-112 showing the greatest reduction, a result that also correlates with the extent of reduction in SuSy message and protein (Zrenner et al., 1995). In terms of cell wall composition, there

is also a corresponding progressive reduction in cellulose content in these line, although the reductions are not dramatic, and only for the T-112 line was reduction truly significant, falling to about 27 % of the control line. There was no significant difference in neutral sugar content, and this was also found to be true for analysis of the individual non-cellulosic sugars determined by quantitation of their alditol acetate derivatives by GLC (not shown. Interestingly, there was a corresponding increase in the uronic acid level that paralleled the decrease in cellulose content. This is reminiscent of our previous studies (Shedletzky et al., 1990 and 1992) supported by a previous BARD grant in which we assessed the changes in cell wall composition in cell cultures adapted to growth on the cellulose-synthesis inhibitor 2,6-dichlorobenzonitrile (DCB). In that study, cellulose levels were markedly reduced and there was a corresponding increase in content of uronic acid-rich polymers.

These results do suggest at least some role for SuSy in providing carbon for cellulose synthesis in potato. The effect was certainly not dramatic, and this perhaps may be explained by the known flexibility of plant cells to adapt to use of alternate pathways and/or that a large proportion of the total enzyme pool for SuSy may be indispensable (see discussion in another recent work of ours where this is discussed with respect to starch synthesis in maize; **Chourey et al., 1998**), and the low residual level is sufficient to support a substantial rate of cellulose synthesis. One aspect we have not yet had time to explore is whether the ratio of membrane-associated SuSy (M-SuSy) to soluble SuSy (S-SuSy) may be altered in these anti-sense lines. The results presented here with the tubers have not yet been submitted for publication, as we are still debating whether the effects are sufficiently large to merit publication.

c) **How many genes for SuSy? Does a specific member of the SuSy gene family associate with the membrane? If not, what does control membrane localization?**

In maize, it seems fairly clear that there are only 2 genes for SuSy—SS1 and SS2 encoded by the *Sh1* and *Sus1* loci respectively. SS1 is expressed predominantly in endosperm whereas SS2 is more abundant in vegetative tissues. However, this is an oversimplification since SS2 can also be found in the basal part of the endosperm, and maize is also rather flexible in patterns of expression, since ectopic expression of SS1 is induced when SS2 protein is lost in a *sus1* mutant (Chourey and Taliercio, 1994). The result cited above (Carlson and Chourey, 1996) that both SS1 and SS2 can be found in membrane-associated forms argues that regulation of membrane association is most likely not due to use of a specific gene product for this function. In further studies relating to this topic, Chourey et al., 1998; see attached manuscript for details), PC's group has studied a double mutant *sh1/sus1*, and compared it to *Sh1/Sus1*, *sh1/Sus1*, and *Sh1/sus1* in terms of the shrunken phenotype, starch content, and levels of SuSy mRNA and enzyme activity. It is clear that the *sus1* mutant is leaky, which may account for a lack of dramatic effect on plant development by the double mutant. These studies also support the notion that the shrunken phenotype is not a result of starch deficiency *per se*, but more likely due to effects on cell wall synthesis, with the SS1 protein being perhaps more important than SS2 for this effect.

The determination of the number of SuSy genes in cotton is complicated by the fact that commercial varieties of *G. hirsutum* and *G. barbadense* are allotetraploids. Southern analyses (Ruan et al., 1997; see attached manuscript for details) suggest that

a small gene family might exist in these species. Fairly extensive screening of DD's cotton fiber cDNA library did not turn up any full-length clones for SuSy but did allow selection of two cDNAs that, when overlapping regions are combined could together encode a full-length SuSy. However, careful analyses show that there are minor nucleotide (and amino acid) differences in the overlapping regions of the cDNA encoding the N-terminal portion of the gene and that encoding the C-terminal portion. Thus, there are certainly at least 2 genes expressed in fibers, but their great similarity suggests the possibility that they might be alleles of the same gene derived from the A and D diploid genomes that have combined to form the allotetraploid. Technician Pat Hogan and Post-doc Liang-Cai Peng in DD's lab have made a construct that fuses these two genes together to make one full-length SuSy cDNA, and we are using this for some of our phosphorylation studies. In addition, Mr. Hogan has recently done RT-PCR using fiber mRNA and has now generated several candidate full-length cDNAs for SuSy that are currently being sequenced. Perhaps the most relevant information at present we have for cotton has come from the recent studies of Dr. Milka Datcheva in DD's laboratory. In the course of our studies on SuSy phosphorylation (see below), we have generated tryptic peptide profiles of both M- and S-SuSy separated by HPLC. The profiles of these peptides are virtually identical with the exception of one peak in M-SuSy that is much more abundant than any other peak. When this was sequenced, it yielded no amino acids, and we presume it was some non-peptide contaminant in the system. Thus, we tentatively conclude that the same gene encodes both forms of the enzyme, consistent with the results from maize.

These results suggest that some other factor(s) controls membrane association. Dr. Datcheva in DD's laboratory has initiated studies on the possible role of phosphorylation in membrane association. Several groups (Huber et al., 1996; Zhang and Chollet, 1997) have now shown that SuSy can be phosphorylated on a specific serine residue in the N-terminal region of the protein. In both maize and soybean this was studied using soluble SuSy as *in vitro* substrate, and evidence indicates that, at least *in vitro*, the major enzyme responsible for this phosphorylation is a form of calcium-dependent protein kinase (CDPK). Most intriguing are recent results from Huber's group (Winter et al., 1997) showing that the percent of M-SuSy rises 10-fold when corn pulvini are gravistimulated and induced to undergo cell elongation. In these studies, this group also suggests that it is the non-phosphorylated form of SuSy that associated with the membrane.

In cotton, we have also shown that S-SuSy can be similarly phosphorylated in a Ca^{2+} -dependent manner by a soluble kinase (Figure 4). Of great interest is the fact that the EGTA-washes of cotton fiber plasma membranes, that contain the eluted M-SuSy, also contain a kinase activity that can phosphorylate M-SuSy in a Ca^{2+} -independent manner (Figure 4). This raises the interesting possibility that differently regulated kinases might function to control localization. Furthermore, other results of ours suggest that the model of Huber's group that suggests that the non-phosphorylated form of SuSy is in the membrane are probably not correct, at least in cotton fibers. Thus, we have shown by *in vivo* labeling studies that S-SuSy and M-SuSy are roughly equally phosphorylated (Figure 5). We have also shown that virtually all of the phosphorylation of either S- or M-SuSy either *in vitro* or *in vivo* occurs on serine residues. Work now is concentrating on

analyzing peptide maps of the phosphorylated peptides. Preliminary results suggest that there are differences between these maps for the two forms phosphorylated *in vitro*. Further complicating the issue is that the maps for these peptides obtained from *in vivo* phosphorylation differ from those obtained *in vivo*, showing that it is not always simple to extrapolate from *in vitro* experiments. Our current approach is now directed at using recombinant SuSy, purified as a his-tagged variant from *E. coli*. We know it is phosphorylated in the *in vitro* system. We shall now check if it serves equally well as substrate for the soluble CDPK and for the other kinase eluted from membranes by EGTA. We shall also be checking how phosphorylation affects ability to reassociate with membranes. We hope these studies will be ready for publication within the next six months. We have learned a lot about phosphorylation, but, at this point, it is difficult to say whether it plays any significant role in membrane localization.

d) SuSy Expression in Relation to Diverse Patterns of Carbon Partitioning in Developing Cotton Seed.

This work was a collaborative effort in which DD's group supplied a cDNA clone of cotton fiber SuSy, and PC's group used it to study the expression patterns of SuSy gene(s) in developing cotton seeds (Ruan et al., 1997; see attached reprint for details). In sum, this work made the surprising finding that, in contrast to that found for other plants, suSy does not appear to play a role in starch synthesis in the cotton seed. The results further strengthened the concept for a role for SuSy in cellulose synthesis, not only by demonstrating high expression of the SuSy gene in the fiber, but also in the transfer cells of the seed coat of the cotyledon which undergo intense wall thickening during development. (High expression in the fiber has also been documented by Nolte et al.,

1995). This SuSy may also be important for degradation of sucrose for the high energy demands required to generate the proton gradient needed for sucrose efflux to the seed apoplast. SuSy was also highly-expressed in the storage cells of the cotyledons where it presumably plays a role in general carbon metabolism needed for synthesis of storage protein and lipid. In sum, this study provides an excellent example of the diverse roles played by SuSy in carbon metabolism.

e) SuSy in a fiberless mutant of cotton.

Fiber cell initiation in the epidermal cells of cotton ovules represents a unique example of trichome development in higher plants. Little is known about the molecular and metabolic mechanisms controlling this process. PC's group has conducted a comparative analysis of the normal (*FLS*) and a *fiberless seed (fls)* mutant (lacking fibers) in cotton to better understand the initial cytological events in fiber development. In addition, carbohydrate analyses were done on developing seeds of the two genotypes, 10 to 35 DAA, to analyze metabolic changes associated with the loss of a major sink for sucrose utilization in cellulose biosynthesis in the mutant.

Our data (Ruan and Chourey, manuscript submitted) so far show that on the day of anthesis (0 DAA), the mutant ovular epidermal cells lacked the typical bud-like projections that are seen in *FLS* ovules and are required for commitment to the fiber development pathway. Gene expression analyses at the cellular level show undetectable levels of sucrose synthase (SuSy) RNA and protein in *fls* ovules, coincident with the stage of fiber initiation. Tissue level analyses of developing seeds, 15 – 35 DAA, have shown an altered temporal pattern of SuSy expression in the mutant relative to the normal

genotype. Whether the altered programming of SuSy expression is the causal or the resultant effect of the mutation is unknown.

The developing seeds have also shown several correlated metabolic changes in the mutant relative to the normal. The most striking changes in the mutant were in the reduced levels of sucrose in seed coat throughout development, and undetectable levels of starch in cotyledons in the early developmental phases. These changes are presumably secondary in nature, as a feed back effect, due to the lack of sucrose utilization in fiber initiation and development in the mutant.

f) Isolation and Characterization of the First Plant Gene Known to Encode the Catalytic Subunit of Cellulose Synthase.

This discovery involved work that we did not propose at all in our initial grant proposal. However, when, in the course of a collaboration with Dr. Dave Stalker and his colleagues at Calgene Inc., two cDNA clones from DD's cotton fiber library were sequenced, they showed some tempting homology to the CelsA genes in bacteria that are known to encode the catalytic subunit of the cellulose synthase. Since no such genes had ever been found in plants, we "dropped everything" for a year and used BARD funding to initiate work on this exciting finding which resulted in a publication that has been widely cited (Pear et al., 1996; see attached reprint for further details) and opened the way for the cloning of similar genes in Arabidopsis (Cutler and Somerville, 1997; Arioli et al., 1998), corn (our work with Pioneer HiBred), rice (databank searches), and tobacco pollen (A. Basic, personal communication). The Calgene group showed that these two genes were highly-expressed in the fiber just at the time the rate of cellulose synthesis increases over 100-fold during development. Southern analysis indicates this is at least a small

gene family in cotton, and perhaps a fairly large one in both *Arabidopsis* and corn (unpublished). Our group did all the sequence comparisons to confirm homology with the bacterial genes, and also identified unique plant-specific insertions in the gene not found in the bacterial counterparts. Dr. Yasushi Kawagoe in DD's lab also subcloned the central portion of the gene which our analyses indicated contained conserved motifs for binding of substrate and catalysis. The subcloned portion was then used to obtain recombinant protein in *E. coli* that was shown to bind UDP-glc (Pear et al., 1996).

The continuation of studies on this gene family is now being supported by other sources, but relative to this grant, we have been studying by use of both the yeast two-hybrid system and direct interaction studies with recombinant proteins, whether any portion of the CelA protein might interact with SuSy. To date, we have not found any interaction, but have still not tested all regions of the proteins, nor all possible conditions.

g) Review Articles.

During the time of this grant, DD has written two review articles relevant to this work (Delmer and Amor, 1995; Kawagoe and Delmer, 1997).

IV. Description of the Cooperation.

The PI and Co-PI maintained contact and cooperation throughout the period of the grant. PC visited DD's lab in Israel during the first year of the grant, and both met again several times at scientific meetings. We exchanged numerous ideas and information concerning research goals and progress and kept in touch frequently by phone and e-mail. Although most publications were not joint, one was (Ruan et al., 1997), and the others were all related to the original and/or modified goals of the proposal.

V. Evaluation of the Research Achievement.

As for most projects, some of our original goals were completed as anticipated, and some proved more difficult than expected. In addition, some new and unexpected findings also led us in some directions different than we had originally predicted. We were not able to determine the cell wall compositions of the wild-type and mutant endosperms of maize, but instead did carry out similar work with potato, with the results supporting the notion that SuSy is important for cell wall synthesis, and that cellulose content is reduced when SuSy levels are lowered by introducing an anti-sense construct of the gene that was expressed in potato tubers. Furthermore, other work indicated that mutant SS1 protein may be the one responsible for the shrunken phenotype in *sh1* mutants, and that this is most likely due, not to reduction in starch content, but in effects upon wall synthesis (Chourey et al., 1998). Consistent also with an important role for SuSy in fiber development is our finding that SuSy and sucrose metabolism are greatly altered in seeds of a fiberless mutant of cotton.

The discovery of a membrane-associated form of SuSy in cotton fibers (Amor et al., 1995) has been highlighted in a specific review article entitled "SuSy ergo GluSy: New Developments in the Field of Cellulose Biosynthesis (Robinson, 1996). It has also opened the way for similar discoveries and work on M-SuSy in maize by PC's group (Chourey and Miller, 1995; Carlson and Chourey, 1996) and by Huber's group (Winter et al., 1997). This work has stimulated an active search for the role of protein phosphorylation in membrane localization by our own group in cotton (unpublished work cited above) and by the Huber group on maize (Winter et al., 1997). In addition, the

work stimulated a group in Japan to engineer a bean SuSy gene into the bacterium *Acetobacter xylinum*, and this has resulted in a remarkable enhancement of cellulose synthesis in vivo (T. Hayashi, personal communication).

The information provided in the above publications resulting from this grant was definitely derived from our original research goals. In addition, the further collaborative work between our two groups that studied the expression patterns of SuSy gene(s) in cotton seeds, shed new light and clearly emphasized the amazingly-diverse roles that SuSy can play in carbon metabolism in plants.

Finally, the exciting and unexpected discovery of the CelA genes of plants was truly a ground-breaking effort that has been high-lighted in several reviews such as “New Hopes for Old Dreams: Evidence that plant cellulose synthase genes have finally been identified” (Haigler and Blanton, 1996), “Cellulose synthesis: Cloning In Silico” (Cutler and Somerville, 1997), “Plant Cell Expansion: scaling the wall” (Nicol and Hofte, 1998), and “A hot mutant for cellulose synthesis” (Delmer, 1998). It has certainly opened a whole new approach to the field of cellulose biosynthesis.

VI. Conclusions.

The work resulting from the this grant leads to a number of conclusions. SuSy clearly plays diverse roles in carbon metabolism. It can associate with the plasma membrane of cells undergoing rapid cellulose deposition, such as cotton fibers, developing maize endosperm, gravistimulated pulvini, and transfer cells of the cotton seed. When SuSy levels are lowered by mutation or by anti-sense technology, cell walls undergo degeneration (maize endosperm) and show reduced levels of cellulose (potato

tubers). In sum, our evidence has very much strengthened the concept that SuSy does function in the plasma membrane to channel carbon from sucrose via UDP-glucose to the cellulose synthase complex. Soluble SuSy also clearly plays a role in providing carbon for starch synthesis and respiration. Surprisingly, we found that the cotton seed is one unique case where SuSy apparently does not play a role in starch synthesis. Current evidence in sum suggests that no specific SuSy gene encodes the membrane-associated form, although in maize the SS 1 form of SuSy may be most important for cell wall synthesis in the early stages of endosperm development. Work is still in progress to determine what does control membrane localization—and the current evidence we have favors a role for Ca^{2+} , and possibly also protein phosphorylation by differentially regulated protein kinases. Finally, we have discovered for the first time, a major new family of genes that encode the catalytic subunit of the cellulose synthase of plants—a result that has opened many new approaches for the study of this important plant function.

VII. List of Publications Resulting from this Grant.

1. Amor, Y, Haigler, C.H., Johnson, S., Wainscott, M., and Delmer, D.P. (1995) A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. *Proc. Natl. Acad. Sci. USA* 92: 9353-9357. 10.2
2. Chourey, P.S. and Miller, M.E. (1995) On the role of sucrose synthase in cellulose and callose biosynthesis in plants. In: *International Symposium on Sucrose*

Metabolism. HG Pontis, EJ Echeverria, eds., American Society of Plant Physiologists, pp. 80-87. ◊

3. Delmer, D.P. and Amor, Y. (1995) Cellulose biosynthesis. *The Plant Cell* 7: 987-1000. — 9.5

4. Carlson, S.J. and Chourey, P.S. (1996) Evidence for plasma membrane-associated forms of sucrose synthase in maize. *Mol. Gen. Genet.* 252: 303-310. ◊ 2.6

5. Pear, J.R., Kawagoe, Y., Schreckengost, W.E., Delmer, D.P., and Stalker, D.M. (1996) Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. *Proc. Natl. Acad. Sci. USA* 93: 12637-12642. ◊ 10.2

J 6. Ruan, Y-L., Chourey, P.S., Delmer, D.P., and Perez-Grau, L., (1997) The differential expression of sucrose synthase in relation to diverse patterns of carbon partitioning in developing cotton seed. *Plant Physiol.* 115: 375-385. ◊ 3.7

7. Kawagoe, Y. and Delmer, D.P. (1997) Pathways and genes involved in cellulose biosynthesis. *Genetic Engineering* 19: 63-87. —

8. Chourey, P.S., Taliercio, E.W., Carlson, S.J., and Ruan, Y-L. (1998) Genetic evidence that the two isozymes of sucrose synthase are critical, one for cell wall integrity and the other for starch biosynthesis in developing endosperm of maize. *Mol. Gen. Genet.*: in press. ◊ 2.6

9. Ruan, Y.-L. and Chourey, P.S. A fiberless seed (*fls*) mutation in cotton is associated with fiber cell initiation in ovule epidermis, and alterations in sucrose synthase expression and carbon partitioning in developing seeds. *Plant Physiology* (Revised copy submitted).

10. (a manuscript on the work on phosphorylation of SuSy will most likely be submitted for publication in fall of 1998)

VIII. Patents (none)

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Figure 1. Carbon partitioning in control and SuSy Anti-sense potato tubers. Figure shows starch content as well as content of cellulose, uronic acids and non-cellulosic sugars in isolated cell walls (see Shedletzky et al., 1992 for methods). Tubers were kindly provided by Dr. Uwe Sonnewald. The three transgenic lines are T-99, T-129, and T-112. See Zrenner et al. (1995) for further details on these constructs.

Figure 2. SDS-NuPAGE separation of cotton fiber proteins that had been isolated in extraction buffer containing 5 mM EGTA. CB: Coomassie Blue staining of gels; WB: Western blotting of proteins. The figure shown was created graphically by combining the results of Western blots that were probed with antibodies against SuSy (bean), β -tubulin (commercial monoclonal antibody), annexin (cotton fiber), 14-3-3 (from Rob Ferl, monoclonal antibody) and cotton fiber Rac13. Plasma membranes were isolated as described by Andrawis et al., 1993. In order to precipitate proteins from the supernatant, CaCl_2 was added to the supernatant with stirring in cold to give a final free Ca^{2+} concentration of 5 mM. The precipitate was collected by centrifugation after incubation in Ca^{2+} for 30 minutes.

Figure 3. SDS-NuPAGE of Cotton Fiber Proteins isolated in presence of 200 nM free Ca^{2+} . With the exception of changes in Ca^{2+} concentration in the initial extraction buffer, all other conditions were the same as for Figure 2.

Figure 4. In vitro phosphorylation of SuSy using extracts prepared from cotton fibers harvested 21 days post-anthesis (stage of active secondary wall cellulose synthesis). Following removal of cell walls by low-speed centrifugation, extracts were separated into membrane and soluble forms by centrifugation at 100,000 g for 1 hr. The membrane fraction was then extracted with 5 mM EGTA to release M-SuSy and associated kinase activity. The phosphorylation shown relied upon endogenous kinase(s). Kinase reactions contained 1-3.3 uCi of γ - ^{32}P -ATP, 5 mM MgCl_2 , buffer, 0.5 uM microcystin, CaCl_2 and EGTA, and unlabeled ATP as indicated and were incubated 10 minutes at 30 C. Following termination with EDTA and addition RIPA buffer, SuSy was immunoprecipitated by addition of anti-SuSy IgG (polyclonal anti-SuSy against bean SuSy; Susy (a generous gift of Dr. Heather Ross) attached to Protein A Sepharose beads. After incubation overnight at 4C, SuSy was precipitated by addition of Protein A beads, washed well in RIPA buffer then PBS and then analyzed by SDS-PAGE. CB: Coomassie Blue Staining.

Figure 5. *In vivo* phosphorylation of SuSy in Cotton Fibers. Fertilized cotton ovules with associated fibers were cultured as described by Haigler et al., (1991). *In vivo* labeling with ^{32}P -orthophosphate (0.5 mCi/mL culture medium) was carried out in medium lacking phosphate for 24 hrs. at 30C. using ovules/fibers of age 21 days post-anthesis. The cultures were starved for phosphate 24 hrs prior to addition of label. Radiolabeled SuSy was isolated by procedures as described in Figure 4 except that the

initial extract contain inhibitors of protein phosphatases (0.1 mM each of NaF and sodium vanadate).

Carbon Partitioning in Control and SuSy Anti-sense Potato Tubers

Nu PAGE of cotton fiber proteins isolated in presence of 5mM EGTA (No free Ca^{2+})

In vitro Phosphorylation and Immunoprecipitation of SuSy

A. Soluble Proteins

B. Membrane Proteins Released by EGTA

SuSy

In vivo Phosphorylation and Immunoprecipitation

Genetic Evidence That the Two Isozymes of Sucrose Synthase are Critical, One for Cell Wall Integrity and the Other for Starch Biosynthesis in Developing Endosperm of Maize.

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Key words: *Zea mays*, endosperm, starch biosynthesis, cellulose, gene expression

ABSTRACT

In maize, two paralogous genes, *Sh1* and *Sus1*, encode two biochemically similar isozymes of sucrose synthase, SS1 and SS2, respectively. Previous studies have attributed the mild starch-deficiency of the *shrunk1* (*sh1*) endosperm to the loss of the SS1 isozyme in the mutant. Here we describe the first mutation of the *sucrose synthase1* (*Sus1*) gene, *sus1-1*, and the isolation of a double recessive genotype, *sh1sus1-1*. Combined data from diverse studies, including Northern, Western, RT-PCR and genomic PCR cloning and sequencing in the 3' region, show that the mutant *sus1-1* gene has both a complex pattern and much reduced levels of expression as compared to the *Sus1* gene. Endosperm sucrose synthase activity in *sh1sus1-1* was barely 0.5% of the total activity in the *Sh1Sus1* genotype. Significantly, comparative analyses of *Sh1Sus1*, *sh1Sus1* and *sh1sus1-1* genotypes have, for the first time, allowed us to dissect the relative contributions of each isozyme in endosperm development. Starch contents in endosperm of the three lineage-related genotypes were 100, 78 and 53%, respectively. The anatomical analyses, including the previously described early cell degeneration unique to the *sh1Sus1* endosperm, yielded no detectable difference between the two *sh1* genotypes. We conclude that the SS1 isozyme performs a predominant role in providing the substrate for cellulose biosynthesis, whereas, the SS2 protein is needed mainly for generating precursors for starch biosynthesis.

INTRODUCTION

The enzyme sucrose synthase (UDPG: D-fructose 2-glucosyltransferase, EC 2.4.1.13) catalyzes a reversible conversion of sucrose and UDP to UDP-Glc and fructose; and plays a major role in energy metabolism by mobilizing sucrose into diverse pathways relating to metabolic, structural, and storage functions of plant cells. The enzyme is well analyzed in many plant species, starting from the first biochemical purification and characterization studies in mung bean (Delmer, 1972) to the most recent analyses of gene expression in barley and peas (Guerin and Carbonero, 1997; Dejardin et al., 1997, respectively). However, the most detailed analyses on this enzyme have been done in maize. Briefly, the two biochemically similar isozymes, SS1 and SS2, are encoded by the *shrunk1* (*Sh1*) and *sucrose synthase1* (*Sus1*) loci, respectively (Chourey, 1981; Echt and Chourey, 1985). Both *Sh1* and *Sus1* loci are cloned and shown to be molecularly homologous (Werr et al., 1985; McCarty et al., 1986; Gupta et al., 1988). Furthermore, both biochemical and molecular data indicate that there are only two SS genes in the maize genome (Chourey et al., 1988; Shaw et al., 1994). A similar system of two or more genes for SS isozymes is also described in sorghum (Chourey et al., 1991b), sugarcane (Buczynski et al., 1993), wheat (Marana et al., 1988), potato (Fu and Park, 1995), Arabidopsis (Martin et al., 1993), sugarbeet (Hesse and Willmitzer, 1996), tomato (Wang et al., 1994) carrot (Sebkova et al., 1995), and barley (Guerin and Carbonero, 1997).

The *Sh1*-encoded SS1 protein is present in most abundant levels in developing

endosperm and a mutational loss of this isozyme is the causal basis of endosperm-specific *shrunk1* seed phenotype. Although the SS1 protein is present in other plant parts, including seedling roots and shoots (Springer et al., 1986; Chourey et al., 1986) no other phenotype is associated with the *sh1* mutation. The *Sus1*-encoded SS2 protein is also detected in many plant parts, including developing embryo (Chourey, 1981; Echt and Chourey, 1985), metabolic sinks such as meristematic tips of young roots and shoots (Chen and Chourey, 1989; Rowland et al., 1989; Nguyen-Quoc et al., 1990), and the aleurone cell layer and basal part of the developing endosperm (Chen and Chourey, 1989; Heinlein and Starlinger, 1989). Unlike the large number of *sh1* mutants, there is only a single mutant of the *Sus1* gene that was first identified on the basis that it lacked the SS2 protein (Chourey et al., 1988). The mutant was discovered while analyzing the SS expression patterns of *Activator (Ac)*-induced *Sh1* revertants reported by Courage-Tebbe et al. (1983). Interestingly, there is no associated phenotypic change with the mutation even though the *Sus1* locus has a unique tissue and cell specific pattern of gene expression. Subsequent genetic analyses to better understand a possible basis for the apparent dispensability of the *Sus1* locus have led to the significant observation that a lack of the SS2 protein in the mutant leads to ectopic activation of the *Sh1* gene (Chourey and Taliercio, 1994). Because the two SS isozymes are biochemically similar (Echt and Chourey, 1985), including an 83% level of similarity in their deduced amino acid sequences (Huang et al., 1994), the ectopically induced SS1 protein in embryos must compensate physiologically for the lost SS2 protein. We attribute such metabolic cross regulation as a basis for the lack of phenotypic change in this mutant.

In this study, we report results from two distinctive but mutually complementary objectives. First, we define the molecular basis of the above-described mutant *Sus1* gene, designated here, *sus1-1*. Gene expression analyses in a double recessive genotype, *shlsus1-1*, indicate that the *sus1-1* allele was complex and leaky in expression. Second, and more importantly, comparative analyses of developing endosperms of the *ShlSus1*, *shlSus1*, and *shlsus1-1* genotypes have allowed us to dissect the relative contributions of each of the two isozymes in developing endosperm. Collectively, the data suggest that the *Shl* gene product performs a critical role in providing the substrate for cellulose biosynthesis, whereas, the *Sus1* product was needed for generating precursors for starch biosynthesis.

MATERIALS AND METHODS:

Maize strains:

Isolation of a double recessive, *shlsus1-1*, genetic stock was previously described (Chourey, 1988). Briefly, *shl* kernels were selected from an F2 ear obtained by selfing dihybrid *ShlshlSus1sus1-1* plants (where the *shl* allele represents a *Shl*-deletion strain, described above). The double recessive, *shlsus1-1*, and the single recessive, *shlSus1*, were indistinguishable by the seed phenotype, but they were separable by genomic Southern blot analyses of DNAs from individual seedlings. The wild type and the mutant segregants yielded one (~16 kb) and two (~23 and 8.5 kb) *Sus1*-hybridizing EcoR1 fragments, respectively, as shown previously (Chourey et al., 1988; Chourey and Taliercio, 1994). Subsequent confirmatory tests on these genotypes

included SDS Western blot analyses with the SS2 monoclonal antibodies on endosperm extracts which showed either a single polypeptide or no detectable SS2 protein for the *Sus1* and the *sus1-1* homozygotes, respectively (see below).

All maize strains were in the W22 inbred background.

Immature kernels at 21 days after pollination (DAP) were harvested, quick frozen in liquid N₂, and stored at -80 C until ready to use.

Protein blot and enzyme activity assays:

Crude extracts from excised endosperm or embryos were prepared as described (Chourey et al., 1986; Chourey and Taliercio, 1994). Protein concentrations were estimated using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules CA). BSA was used as the standard. Samples were electrophoresed on a 4.5 to 7.0% linear gradient nondenaturing gel, electroblotted to nitrocellulose, and immunostained using the appropriate primary antibody as described previously (Chourey et al., 1986).

Denaturing Western blots were done as described previously (McElfresh and Chourey, 1988). Specificities of the polyclonal and monoclonal SS and AGPase antibodies are described previously (Echt and Chourey, 1985; Chourey et al., 1991b; Giroux and Hannah, 1994) Antibody binding was detected via chemiluminescence using the SuperSignal CL-HRP Substrate System detection kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Sucrose synthase activity was measured as rate of sucrose cleavage, as previously described (Chourey, 1981). All measurements were performed in triplicate. Prior to enzyme assays, extracts were dialyzed overnight at 4° C against 5 mM Tris-

maleate and 0.1 mM DTT buffer pH 7.2. Alternatively, several assays were also performed using extracts passed over Micro Bio-Spin 6 columns ((Bio-Rad, cat. # 732-6221) to remove endogenous sugars from crude extracts, according to manufacturer's instructions. The latter method was particularly useful for extracts from the double mutant as it reduced the time between extraction and the enzyme assay to less than two hours.

Starch measurements

Starch determinations on 21 DAP kernels were done using a starch assay kit (#207748, Boehringer Mannheim, Indianapolis, IN), according to the manufacturer's instructions. Briefly, starch was extracted using dimethyl sulfoxide and hydrolysed to glucose by amyloglucosidase based on a starch assay kit. Starch levels were measured as glucose equivalents and expressed according to the values from a set of starch standards.

RNA, RT-PCR and Genomic PCR analyses

Total RNA was isolated from 21 DAP endosperm as described (Wadsworth et al., 1988). Poly (A)+ RNA was isolated by two cycles of adsorption to elution over an oligo(dT)-cellulose column (Maniatis et al., 1982). Ten-microgram samples of total RNA were glyoxalated, fractionated on a 1.2% agarose gel and transferred to a Nytran membrane. The blots were prehybridized in 50 mM PIPES, pH6.5, 100 mM NaCl, 50 mM sodium phosphate buffer, pH 6.5, 1 mM EDTA, and 5% SDS. The blots were hybridized in the same solution with 3×10^6 cpm/ml of radiolabelled probe overnight at 65°C. Blots were rinsed at 65°C 2x for 45 min in 6xSSC (1 x SSC is 0.15 M NaCl,

0.015 M sodium citrate), 5 mM EDTA, pH 8.0, 5 mM sodium phosphate, pH 6.5, 5% SDS and 2x for 30 min as above except in 0.2xSSC for the *SusI* and *Sh2* probes.

The primers were based on the cDNA sequence (Huang et al., 1994), and included: #21 (5'-CATCACCAGTACCT TCCAAG-3');
 #140 (5'-GCCTACGGCGGTCCGGCCGAGATC);
 #141 (5'-CGTCACGCATCACTTGCTGGAGG); and oligo(dT) (5'-GCGGATCC (T)₁₈).
 First strand cDNAs were synthesized from 10 µg of total RNA using either oligo d(T) or primer #141 as the primer and Superscript II (BRL) at 42°C following the manufacturer's instructions. PCR amplification was performed on 2 µl of the cDNA solution in a 100 µl volume using *Taq* polymerase following the manufacturer's instructions. Genomic PCR amplification was based on 8 µg of DNA (Dellaporta, et al., 1983) from *susI-1* etiolated seedlings as template and the primer pair #21 - #141. An aliquot of 5 µl from the first PCR reaction was later used as a template in the second round of amplification using the #140 - 141 primer pair. A ~800 bp fragment was cloned and sequenced. A "touchdown program" (Don et al., 1991) was used for all PCR reactions. It consisted of four cycles of a 1 min denaturation at 94°C, a 2 min annealing step at decreasing temperatures followed by a 3 min extension step at 72°C. The annealing step started at 66°C and decreased by two degrees to 48°C. The program finished with a 10 min extension at 72°C. NaCl was added to an aliquot of the PCR reaction to 10 mM, and the products were separated on a 1% agarose gel where they were visualized and blotted by standard methods.

Construction of cDNA library

A cDNA library was made from *sus1-1* kernel poly(A)⁺ RNA using the ZAP cDNA Synthesis kit (Stratagene cat. #200403) following manufacturer's instructions. First strand cDNA was primed using an oligo (dT) primer with an *Xho*I site added at the 5'-end and synthesized using reverse transcriptase in the presence of 5-methyl dCTP. The second strand of the cDNA was primed by RNA fragments remaining after RNase H treatment of the DNA:RNA duplex and synthesized by DNA polymerase 1. The DNA was blunt-ended with Klenow fragment and *Eco*R1 adaptors were ligated onto the DNA. Digestion of the DNA with *Xho*I removed adaptors from the 3'-end of the cDNA. Larger DNA fragments were isolated on a Sephacryl spin column and ligated into the Uni-ZapXR vector and packaged using GigapakII gold packaging extracts (Stratagene cat. #200214).

Sus1 cDNAs were identified by screening 5×10^5 pfu of the cDNA library with the *Sus1* radiolabelled cDNA clone (Gupta et al., 1988) following standard protocols. Plasmids containing the *sus1-1* inserts were recovered by *in vivo* excision. Sequence analyses were limited to the 3' end, 1470 to 2559 bp, of a 2.4 kbp clone because previous genomic Southern data indicated that the rearrangement is at this end of the gene (Chourey et al., 1988).

Preparation of sections and microscopy

Immature kernels of various genotypes at 12 DAP were fixed in formalin acetic acid, dehydrated through a tertiary butyl alcohol series, cast into paraffin blocks and processed as described (Chen and Chourey, 1989). Paraffin sections were also used for scanning electron microscopy (SEM). Using a coplin jar, the sections were de-

paraffinized in 100% xylene for 30 to 40 min, followed by 100% acetone (10 min, 2X) and finally 100% ethanol (10 min). As an alternative to critical point drying, samples were treated with the solvent hexamethyldisilazane (HMDS, Polysciences #00692, Warrington, PA, USA). The slides were covered with HMDS for 2 to 5 min and placed in a drying oven overnight to remove HMDS. The slides were then scored with a diamond knife and individual kernel sections mounted on specimen stubs using double-stick carbon tabs and graphite suspension. The mounted specimens were sputter coated with gold.

RESULTS

cDNA and genomic cloning, and sequence analysis of the 3' region of the mutant *sus1-1* gene

Figure 1 represents a schematic map of the 3' region and sequence alignments showing the point of divergence of the *Sus1* and *sus1-1* genomic and cDNA clones. These analyses were restricted to the 3' end because previous genomic Southern analyses have indicated that a rearrangement at this end of the gene was the basis of the mutation (Chourey et al., 1988). A 2.4 kbp cDNA clone was isolated from a *sus1-1* cDNA endosperm library and an approximately 1 kbp region at the 3' end was sequenced. Primer #21 at 1470 bp represented a common 5'-end sequence of the two genes whereas primer #141 at 2487 bp at the 3'-end of the gene was unique to the *Sus1* allele (Gupta et al., 1988). A comparison of the cDNA sequences of the *Sus1* and *sus1-*

I alleles (Fig. 1B) indicate that these sequences diverge at the 2363 bp position and include a premature stop codon TAG in the foreign DNA of the *sus1-1* allele. A genomic clone with an insert of ~800 bp was also isolated by PCR amplification using DNA from *sh1sus1-1* etiolated seedlings as a template, and the primers #140 - 141 (Gupta et al., 1988). Sequence comparisons with the *Sus1* gene (Shaw et al., 1994) show an intact 3' end in the mutant *sus1-1* genome (Fig. 1C), and an additional ~350 bp of non-*Sus1* foreign DNA which was inserted 5' to the 2364 bp in the last exon of the gene (sequence data not shown). The non-*Sus1* sequences are submitted to the GenBank, AF043382, and have shown no match to any sequences in the GenBank database.

Fig. 1

Expression analysis of the *sus1-1* mutant allele at RNA, protein and enzyme activity levels

RNA level

Figure 2 illustrates an RNA blot showing steady state levels of the SS2 transcripts in 21 DAP endosperm of the *Sus1* and *sus1-1* genotypes. The normal sized, 2900-nt, SS2 transcript encoded by the wild type *Sus1* gene was detectable in 4 µg of total RNA from the *Sus1* endosperm. No such normal sized transcript was seen in the mutant *sus1-1* RNA; instead, a truncated transcript, ~2500-bp, was seen at reduced steady state levels at only 20 µg or higher loading levels. The quality of RNA in these tissues was comparable based on intensities of ethidium bromide-stained rRNA bands (data not shown), and by hybridizations to maize *shrunk2* clone (*Sh2*, Giroux and Hannah, 1994). Figure 2B

Fig. 2

shows a parallel RNA blot based on 5 μ g of *sh1Sus1* and *sh1sus1-1* endosperm RNA, and hybridized with the *Sh2* cDNA clone. Near equal steady state abundance of the *Sh2* transcripts in the two genotypes was indicative of not only an internal loading control, but it served to show that unlike the antisense inhibition of the SS genes in potato (Zrenner et al., 1995), there was no cross-regulatory interaction between the SS and Adenine diphosphate glucose pyrophosphorylase (AGPase) genes in maize (see below). Furthermore, as shown in Figure 2C, the SDS Western blot on endosperm extracts of the same two genotypes also show a near equal steady state abundance for the 54 kD polypeptide of the *Sh2*-encoded protein, the large subunit of the AGPase protein (Giroux and Hannah, 1994).

RT-PCR analysis

We used the RT-PCR technique to further analyze the expression of the *sus1-1* gene. Figure 3 shows the data from RT-PCR analysis using RNAs from 21 DAP *Sus1* and *sus1-1* kernels as templates and the two primer pairs, #21 - oligo(dT)₁₈ and #21 - #141. Relative positions and orientations of these primers, and their sequences are shown in Fig. 1 and in Materials & Methods, respectively. The *Sus1* mRNA yielded the expected sized, 1.4 and 1.0 kbp, amplified products using the above primers. The *sus1-1* mutant mRNA, however, yielded a smaller fragment, 1.0 kbp, from the #21 - oligo d(T) pair and a slightly larger, 1.1 kbp, but of much reduced state levels, from the #21 - #141 pair.

Fig. 3

Western blot analyses

Figures 4 A and B represent nondenaturing and SDS Western blots, respectively, showing SS2 proteins in endosperm extracts of the *Sus1* and *sus1-1* genotypes. The

nondenaturing blots prepared from endosperm extracts of the *Sus1* kernels show a single SS2 protein band which was cross-reactive to both polyclonal and monoclonal antibodies. In contrast, the *sus1-1* extract did not show the normal SS2 protein band; instead a slow migrating band detectable by only the polyclonal SS2 antibodies was seen. Surprisingly, the monoclonal antibodies did not detect any cross-reactive proteins in the mutant extracts.

The same protein extracts, after denaturation, were also analyzed in SDS Western blots (Fig.4B). A single polypeptide of 87 kD was seen in *Sus1* endosperm extracts by both polyclonal and monoclonal antibodies, as reported previously (Chourey, 1981, Echt and Chourey, 1985). Similar extracts from the *sus1-1* mutant, however, revealed two polypeptides as a major and a minor band (based on band intensity) by the SS2 polyclonal antibodies, but only one of these two proteins was detectable by the monoclonal antibodies. Interestingly, the SS2 polypeptide of the apparent similar size as the normal subunit (the major band) was not reactive with the monoclonal antibodies. In addition, to these qualitative changes, there was an overall reduction in the relative abundance of the SS2 protein in the mutant relative to the levels seen in the normal genotype (note, a 10-fold loading difference in the amounts of protein between the two genotypes).

Sucrose synthase activity

Enzyme activities in 21 DAP endosperm extracts of the two genotypes, *sh1sus1-1*, *sh1Sus1*, in addition to the *Sh1Sus1* stock, are shown in Table 1. Endosperm enzyme activities in the double dominant, *Sh1Sus1*, and the single dominant *sh1Sus1* genotypes represent the cumulative expression of the two loci and only the single *Sus1* locus, respectively, (Chourey and Nelson, 1976; Chourey, 1981). The *sh1Sus1* endosperm

Fig.4

Table 1

extracts show ~8% of the *ShISus1* enzyme activity, as shown previously (Chourey, 1981); however, the double recessive, *shlsus1-1*, endosperm extracts have shown only 0.51% of the *ShISus1*, or ~6% of the *shlsus1*-genotype (Table 1).

Figs. 5 and 6.

Causal effects of the *sus1-1* mutant in developing kernels

Starch levels in the 21 DAP endosperms of the three genotypes, *shlsus1-1*, *shlsus1*, and *ShISus1* are also shown in Table 1. The first two genotypes show significant reductions in starch content, i.e., 46% and 22%, respectively, as compared to the *ShISus1* endosperm. Overall, the *shlsus1-1* endosperm reflects an additional 24% reduction in starch content relative to the *shlsus1* endosperm; there was, however, no detectable change in seed phenotypes as both genotypes exhibit the same degree of the shrunken seed phenotype. A lack of change in seed phenotype was not surprising, a similar situation in an unrelated genotype is also reported by Singletary et al. (1997) where a reduction of ~55% in endosperm starch content relative to the normal levels (100%) has led to no change in the seed phenotype. At the cellular level the two *shl* genotypes were readily distinguishable from the *ShISus1* genotype, as early as 12 DAP, in the longitudinal sections shown in Fig. 5. The intensity of I₂KI staining was indicative of *ShISus1* > *shlsus1* > *shlsus1-1*. More importantly, these kernel sections have shown cell degeneration in both *shlsus1-1* and *shlsus1* kernels, as shown previously for the latter genotype (Chen and Chourey, 1989; Chourey, et al., 1991a). No such degeneration was seen in the *ShISus1* or the *Shlsus1-1* (data not shown) endosperms. Unlike the additive effect on reduction of starch levels in the *sus1-1* relative to *Sus1* endosperm (Table 1), there was no detectable difference in the degree

of cellular degeneration in the two genotypes. It is of interest that the *sus1-1* kernels did not show any degeneration in the basal part of the endosperm because the SS2 protein is predominantly localized to this region (Chen and Chourey, 1989). In addition, scanning electron micrographs of sections of 12 DAP endosperms (Figure 6), representing the same location in all kernel sections, has shown a detectable reduction in the size of starch granules in the two *sh1* genotypes relative to the *Sh1* endosperm. There was however no such size difference between the two mutant genotypes.

DISCUSSION

1. Evidence that the mutant *sus1-1* allele is leaky as well as aberrant in expression

In maize, the two SS transcripts and proteins encoded by the genes, *Sh1* and *Sus1*, are among the most abundant gene products in developing endosperm at 20 -24 DAP stage (Chourey, 1981; Werr et al., 1985). Of the two genes, *Sh1* contributes to >90% of the total SS gene product. Thus, it was essential to isolate a double mutant, *sh1sus1-1*, where the *sh1* allele is null in expression (Chourey, 1981), to allow endosperm level analyses of the mutant *sus1-1* gene which is significantly reduced in expression relative to the normal *Sus1* gene (see below).

We present several lines of evidence to show that the *sus1-1* mutant allele was both leaky (i.e., reduced levels of expression) and encoded products which were distinguishable from those of the wild type, *Sus1*, gene. First, sequence analyses of the largest *sus1-1* cDNA clone have shown a loss of ~500 bases at the 3'-end of the gene. In addition, the

cloned sequences show, starting 2363 bp from the 5'-end, 196 bp of foreign sequences (only a part of these sequences are shown here, Fig. 1B) of unknown origin and significance, as no match was detected in our GenBank database searches. Second, Northern blot analyses on RNAs from *Sus1* and *sus1-1* endosperm have shown reduced steady state levels of a truncated *Sus1* transcripts in the mutant relative to the wild type genotype. Indeed, the RT-PCR data, especially based on the primer pair, #21 - oligo d(T), yielded a smaller fragment, ~1.0 kbp, from *sus1-1* RNA, and not the expected sized 1.4 kbp fragment which was seen from the *Sus1* RNA. It is reasonable to suggest, based on these data, that the truncated transcript seen by Northern blot analyses is due to transcript termination in the non-*Sus* region (note, a TAG codon 7 bp downstream from the point of divergence, Fig. 1B) and was lacking the normal 3' end sequences. In addition, RT-PCR analysis using mutant RNA showed amplification of a slightly larger fragment than that from the *Sus1* RNA template and the primer pair #21 - #141 which included the very terminal translated sequences at the 3'-end of the gene. Indeed, sequence analysis of PCR-amplified and cloned *sus1-1* genomic DNA (Fig. 1C), based on the primers #140 and #141 (Gupta et al., 1988), have shown the complete 3' end, including the #141 primer, of the *Sus1* gene. Thus, the larger transcript was chimeric and may include both the non-*Sus1* sequences and the very 3' *Sus1* encoding sequences which were otherwise missing in the more abundant truncated transcripts. We suggest that the mutant *sus1-1* gene encodes at least two populations of the *sus1-1* transcripts in a developing endosperm. Although our RT-PCR data are not quantitative, the larger transcript was seen at much reduced steady state levels; and it was also not detectable by Northern blot analyses.

Finally, the data from studies on expression at the protein level have shown much reduced steady state levels both by Western blot analyses as well as in enzyme activity in the *sus1-1* mutant as compared to the *Sus1* genotype. Furthermore, there are several observations which suggest that the *sus1-1* locus encoded an aberrant SS2 protein. It is of significant interest that the relatively slow migrating SS2 band in the *sus1-1* extracts detectable by polyclonal antibodies was not reactive to the monoclonal antibodies in nondenaturing Western blots. Clearly, the protein was altered both in its total surface charge (but no apparent change in size based on the SDS Western data), and its immunospecificity presumably due to, among other factors, an abnormal folding of the polymer of two different sized subunits visualized by SDS Western blot analyses. These subunits were also distinguishable in epitope specificities such that only one of the two was reactive with the monoclonal antibodies. It is possible that the nonreactive subunit renders the reactive epitope inaccessible upon heteromerization. Unlike polyclonal antibodies, the epitope-specificity of monoclonal antibodies can be as small as a change in the single amino acid (Pappu et al., 1993) or a single stretch of a few amino acids in a polypeptide (Laver et al., 1990). The larger SS2 subunit of the mutant protein may also have a stretch of several non-SS2 residues.

Sucrose synthase is a homotetramer in all plant species analysed so far, including mung bean, (Delmer, 1972), rice (Nomura and Akazawa, 1973), bamboo (Su et al., 1977), sugarcane (Buczynski et al., 1993), barley (Guerin and Carbonero, 1997) pea (Dejardin et al., 1997) and maize (Su and Preiss; 1978; Echt and Chourey 1985; Chourey et al., 1986, 1988). It is possible that the mutant SS2 protein is also a homotetramer composed of only

the reductions of only ~25% in the former as compared to the latter genotype (Singletary et al., 1997; Table 1, here). Although the *sh1* mutation is the first genetic demonstration that SS enzyme plays a critical role in starch biosynthesis in plants, the recent data suggest that starch deficiency *per se* is not the causal basis of the *shrunkeni* seed phenotype.

One of the most pertinent observations is the current anatomical data on kernel sections of the *sh1Sus1* and *sh1sus1-1* genotype (Fig. 5) which show cell degeneration at 12 DAP. As shown previously on the basis of the *sh1Sus1* kernels (Chen and Chourey 1989; Chourey et al., 1991a), the cell degeneration was spatially restricted to the centrally located starch storage cells, and it occurred *prior* to the onset of the rapid phase of starch biosynthesis which is known to initiate at ~12 DAP (Tsai et al., 1970). It should be noted that starch deficient cells are not inherently susceptible to such degeneration because no such cellular loss is seen in the *sh2* mutant (Chourey et al., 1991a) which accumulates only ~20 to 30% of the wild type levels of starch. Indeed, Young et al. (1997) have now shown programmed cell death mediated degeneration in the *sh2* mutant, starting at 24 DAP. Remarkably, unlike the *sh1* mutant, cell degeneration in the *sh2* mutant occurs near the basal part of the kernel adjacent to the scutellum (Young et al., 1997). Finally, it is significant that the *Sh1*-encoded SS1 isozyme is also developmentally induced at ~12 DAP (Chourey, 1981). Although the SS catalyzes a readily reversible reaction, the sucrose breakdown reaction leading to UDP-Glc synthesis is considered to be the more important *in vivo* function in endosperm (Chourey and Nelson, 1979). Because UDP-Glc is a common precursor for both starch and cellulose biosynthesis, drastic reductions of the SS activity in the two *sh1* genotypes (Table 1) is expected to affect both pathways. This is indeed the case as evidenced by the brittle cell walls and the reduced size of starch grains, Figs. 5 and 6,

respectively. However, it is puzzling that Shannon et al. (1996) did not observe any difference in the levels of UDP-Glc in *Sh1* and *sh1* endosperm, nor is there any explanation at present for the unexpected results. We suggest that much reduced levels of the SS activity in the two *sh1* genotypes, coincident with the cell elongation phase and the onset of starch biosynthesis, must create competing demands for UDP-Glc. We suggest that a rate limiting flux of UDP-Glc in the cellulose biosynthesis during cell elongation initiates the degeneration process. Thus, the collective data suggest that the early loss of cellular sites for starch biosynthesis is the primary, and starch-deficiency the secondary, causal basis for the *sh1* endosperm.

A critical role for the SS enzyme in cellulose biosynthesis is also shown in cotton fiber where the SS protein is tightly associated with plasma membrane, and is hypothesized to 'channel' UDP-Glc to the growing glucan chain more efficiently (Amor et al., 1995). Indeed, the two SS isozymes in maize are also associated with plasma membrane, in addition to being cytosolic (Carlson and Chourey, 1996). Overall, these data are in agreement with the observations of Maas et al. (1990) on transient transformation in maize, showing a blockage of the *Sh1* promoter activity in transfected protoplasts treated with an inhibitor of cellulose biosynthesis.

The reduction in the size of starch grains in both genotypes of *sh1* endosperm relative to the wild type (Figure 6) is in agreement with Shannon et al. (1996) who have presented quantitative data (i.e., number of starch grains / mg starch), on such reductions in starch granule sizes. These authors report that such reductions are a generalized phenomenon common to several starch deficient mutants in maize, albeit, the *sh1* mutant shows the least reduction among the genotypes they studied. We surmise that the reduced

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levels of precursors due to the SS1-deficiency may also contribute to slight reductions in the size of starch grains.

It is important to point out here that there was no detectable difference in the degree of cellular degeneration between the *sh1sus1-1* and the *sh1Sus1* endosperm sections (Fig. 5). Remarkably, the *sus1-1* mutant did not show any degeneration in the basal part of the endosperm where a substantial proportion of the SS2 protein is immunolocalized (Chen and Chourey, 1989). There was, however, a readily detectable difference in the levels of starch content both at 12 and 21 DAP stages by cytological and the quantitative determinations, respectively. Much reduced starch content, i.e., 53% as compared to 78% of the wild type (Table 1), but no change in cellular stability in the former as compared to the latter, implies that the *Sus1*-encoded SS2 isozyme performs a predominant role in providing precursors for starch biosynthesis. A similar role of the SS2 isozyme is also apparent in our comparisons of endosperm starch levels in *Sh1Sus1* and *Sh1sus1-1* genotypes where the latter has ~10% reduction as compared to the former (data not shown).

An overall role of the SS enzyme in starch biosynthesis is consistent with the results based on antisense inhibition of the SS genes in transgenic potato where the transgen with a loss of up to ~98.6% of the SS activity shows an associated reduction of ~66% starch content as compared to the control tubers (Zrenner et al., 1995). Whether or not there is any effect on cellular stability in such transgenic tubers is not known. Interestingly, the transgenic potato line has also lost AGPase expression at the RNA level, but there is no loss of the enzyme activity. The AGPase is known to play a critical role in providing substrates in starch biosynthesis (Preiss, 1988). The cross-regulation between AGPase and

the SS genes is intriguing but it appears to be unique to the transgenic potato lines, as our data on the *shlsus1-1* endosperms have not shown any deviations from the normal levels of the *Sh2*-encoded AGPase transcripts and the protein.

Finally, although both maize and potato studies discussed above, clearly demonstrate that SS enzyme plays a critical role in starch biosynthesis, a substantial level of starch accumulation is seen in both the plant systems despite the very low levels of SS activity in the respective organs. The *shlsus1-1* endosperm retained ~50% starch levels even though there was only ~0.50% of the total SS activity as compared to the double dominant, *Shlsus1*, endosperm. We suggest that either a substantial level of starch biosynthesis in storage tissues occurs independent of the SS enzyme (i.e., metabolic plasticity), or that a very large proportion of the total wild type levels of enzyme activity is dispensable, as shown previously for various enzyme systems in plants (Chourey and Nelson, 1979; Quick et al. 1991; Gottlob-McHugh et al., 1992; Zrenner et al., 1995; Cheng et al., 1996). It should be pointed out, however, that our results here are limited to developing endosperm, and to plant survival only under the normal growth conditions. In this regard the recent data on *shlsus1-1* seedlings are of particular interest as they show clearly that SS is critical for the anoxic tolerance of maize roots (Ricard et al., 1998).

Figure 1. Comparison of *Sus1* and *sus1-1* cDNA and genomic sequences in the 3' region.

A. Relative positions and the orientations of the primers, as shown; and the numbers in bp refer to the coding region (Gupta et al., 1988).

B. Sequences of the RT-PCR derived cDNA clones from the *Sus1* and *sus1-1* endosperm.

No sequence identity was seen beyond 2363 bp, and the underlined codon in bold represents a premature stop codon in the foreign sequences of the *sus1-1* mutant. (Only a portion of the foreign sequences submitted to GenBank, beyond 2363 bp are shown.)

C. Sequences of the genomic *sus1-1* clone are aligned against the *Sus1*, starting at 2364 bp. The # in parenthesis represent genomic #, Shaw et al. (1994). Sequences for the primer # 141 are shown in bold. (Only a portion of the foreign sequences, 5' to the 2364 bp are shown.)

Figure 2. Expression patterns of (A) *Sus1*, (B) and (C) *Sh2* genes in *sh1Sus1* and *sh1sus1-1* endosperms.

(A) RNA gel blot showing *Sus1*-specific transcripts in total RNA of *sh1Sus1* (4 µg) and *sh1sus1-1* (20 µg) genotypes.

(B) RNA gel blot and (C) SDS Western blot, at 5 µg levels each, showing AGPase transcripts and protein in *sh1Sus1* and *sh1sus1-1* endosperm.

Figure 3. RT – PCR analysis of *Sus1* and *sus1-1* encoded transcripts in 21 DAP kernels of genotypes, as shown.

Figure 4. The mutant *sus1-1* locus encodes an aberrant SS2 protein. (A) Non-denaturing

Western blot representing crude endosperm extracts, at protein levels of 2.5 and 25 μ g of *Sus1* and *sus1-1* genotypes, respectively. (B) SDS Western blot representing the samples shown above, at protein levels of 5 and 50 μ g for *Sus1* and *sus1-1* genotypes, respectively.

Figure 5. Longitudinal sections of 12 DAP kernels of the genotypes, as shown, are stained for starch with I₂KI and Fast Green for cellular detail. The bar represents 300 μ m.

Figure 6. SEM micrographs of 12 DAP kernels of the genotypes, as shown.

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Table 1. Sucrose synthase activity (nmol/ mg protein/ min) and starch level (mg/ g FW) in developing maize endosperms at 21 DAP.

Genotype	<u>Specific activity</u>	<u>Starch</u>
<i>sh1sus1-1</i>	15.6 ^a (0.51) ^b	114.8 (53.5)
<i>sh1Sus1</i>	258.5 (8.4)	168.2 (78.3)
<i>Sh1Sus1</i>	3078.0 (100)	214.5 (100)

^a Each value is the mean of at least three replicates with standard error less than 10 % of the mean.

^b The value in the parenthesis represents the percentage of that from *Sh1Sus1*.

Sus1 #21 → #140 → ← #141 ← oligo dT
 1470 bp 2119 bp 2487 bp AAAA

sus1-1 #21 → ← oligo dT
 2363 bp ----- AAAA

2311 bp
↓
Sus1 TCGGAGAGGCTGATGACCCTCACCGGCGTGTACGGGTCTCTGGAAGTAC
.....
sus1-1 TCGGAGAGGCTGATGACCCTCACCGGCGTGTACGGGTCTCTGGAAGTAC
2363 bp
↓
Sus1 GTGTCCAACCTGGAGAGGCCCGAGACCCGGCGGTACCTGGAGATGCTG
.....
sus1-1 GTGTCAGATGCCTAGGTATGTGAAGTTGCATTACCGCCTAGTAGTAGTC

2364 bp (7245 bp)

Sus1 CAACCTGGAGAGGGCGCGAGACCCGGCGGTACCTGGAGAT
.....

sus1-1 ATCACAGTACGTGCCCAACCTGGAGAGGGCGCGAGACCCGGCGGTACCTGGAGAT

Sus1 GCTGTACGCGCTCAAGTACCGCACCATGGCGAGCACCGTGCCCGTGGCCGTGG
.....

sus1-1 GCTGTACGCGCTCAAGTACCGCACCATGGCGAGCACCGTGCCACTGGCCGTGG

Sus1 AGGGAGAGCCCTCCAGCAAGTGATGCGTGACG
.....

sus1-1 AGGGAGAGCCCTCCAGCAAGTGATGCGTGACG

2487 bp

Fig. 2

RT-PCR:

Fig. 3

Primers : 21-oligo dT 21-141
 Sus1 *sus1-1* *Sus1* *sus1-1*

Fig 4

Fig. 5

Fig. 5 (continued)

Sh1Sus1

sh1Sus1

sh1sus1-1

Fig. 6

Sh1Sus1

sh1Sus1

sh1sus1-1