Fructan Accumulation and Sucrose Metabolism in Transgenic Maize Endosperm Expressing a *Bacillus amyloliquefaciens* SacB Gene

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Over 40,000 species of plants accumulate fructan, β-2-1- and β-2-6-linked polymers of fructose as a storage reserve. Due to their high fructose content, several commercial applications for fructans have been proposed. However, plants that accumulate these polymers are not agronomically suited for large-scale cultivation or processing. This study describes the transformation of a *Bacillus amyloliquefaciens* SacB gene into maize (*Zea mays* L.) callus by particle bombardment. Tissue-specific expression and targeting of the SacB protein to endosperm vacuoles resulted in stable accumulation of high-molecular-weight fructan in mature seeds. Accumulation of fructan in the vacuole had no detectable effect on kernel development or germination. Fructan levels were found to be approximately 9-fold higher in *sh2* mutants compared to wild-type maize kernels. In contrast to vacuole-targeted expression, starch synthesis and endosperm development in mature seeds containing a cytosolically expressed SacB gene were severely affected. The data demonstrate that hexose resulting from cytosolic SacB activity was not utilized for starch synthesis. Transgenic seeds containing a chimeric SacB gene provide further evidence that the dominant pathway for starch synthesis in maize endosperm is through uridine diphosphoglucose catalyzed by the enzyme sucrase synthase.

Higher plants contain carbohydrate storage reserves, most commonly in the form of starch (corn, other cereals) but in some cases as Suc (sugar beet, sugar cane) or fructan, a Fru-containing polymer (Jerusalem artichoke, chicory). These reserves are of considerable biochemical and physiological interest and also of great economic importance. This paper reports the results of experiments originally directed at the biosynthesis of fructan in a species in which the polymer does not ordinarily exist, but that unexpectedly also provided a tool that sheds light on basic questions concerning Suc metabolism and starch biosynthesis during corn seed development.

Fructans consist of a terminal Suc residue and linear or branched chains of repeating Fru units. Several food and nonfood applications have been suggested for fructans, due to their high Suc content (Barta, 1993; Fuchs, 1993). Jerusalem artichoke and chicory may be the two crops most suited for large-scale production (Fuchs, 1991; Fontana et al., 1993). However, fructan-containing crops are at a production disadvantage relative to more traditional agricultural crops due to their low yield and poor agronomic performance and the lack of processing technology.

Expression of chimeric FTF genes in traditional crops may overcome these problems. Expression of a chimeric *Bacillus subtilis* FTF, directed by the CaMV 35S promoter, was recently reported in transgenic tobacco and potato (Ebskamp et al., 1994; van der Meer et al., 1994). Targeting the FTF protein, which uses Suc as the sole substrate, to the vacuole resulted in accumulation of fructan in the leaves and microtubers of transgenic plants. Diversion of Suc into fructan also led to decreased starch levels in tobacco leaves and a severe reduction in starch accumulation, dry weight, and number of microtubers formed in transgenic potato (Ebskamp et al., 1994; van der Meer et al., 1994). The results suggest that diversion of Suc from an existing metabolic pathway may be detrimental to tissue development.

Suc is one of the principal products of photosynthesis and is the preferred transport carbohydrate in higher plants. The critical role of Suc in plant growth and development was recently illustrated using transgenic plants expressing a chimeric yeast invertase gene (Sonnewald et al., 1993, 1994). Characterization of endosperm mutants has been invaluable to the study of Suc metabolism and starch synthesis in maize (*Zea mays* L.) (reviewed by Hannah et al., 1980; Duffus, 1993; Nelson and Pan, 1995). However, many questions regarding Suc partitioning and the conversion of Suc to starch in developing kernels remain unanswered. One of these questions concerns the relative roles of the enzymes SS and invertase in the synthesis of starch. Suc, the primary source of carbon for starch synthesis, is hydrolyzed by invertase as it enters the basal region of the endosperm (Shannon 1968, 1972). The resulting hexose is believed to be utilized for resynthesis of Suc in the upper portions of the developing kernel. The resynthesized Suc is converted into Fru and UDP-Glc by SS before entering the starch biosynthetic pathway.

Characterization of mutants at the *Sh* locus, which codes for the SS-1 protein, suggest that SS plays a significant role in the conversion of Suc to starch (Choure and Nelson, 1976). However, deletion mutants of the *Sh* gene result in only a mild reduction, not a complete loss, of starch in mature seeds. The results led to the discovery of a second, nonallelic SS gene, coding for the Sus protein (Echt and

Abbreviations: CaMV, cauliflower mosaic virus; DPP, days post-pollination; FTF, fructosyltransferase; SS, Suc synthase.

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Chourey, 1985; McCarty et al., 1986). The Sus protein was reported to be active at very low levels in the endosperm. Although the low level of Sus expression could account for the moderate levels of starch in sh mutants, it is also possible that a significant amount of starch could be synthesized by a pathway independent of SS. Felker et al. (1990), using maize endosperm tissue slices, also suggested that hexose may enter the starch biosynthetic pathway, but tissue slices may not necessarily represent in vivo conditions. The questions of whether hexose is phosphorylated and then converted to starch in the amyloplast or if UDP-Glc is a critical metabolite in the starch biosynthetic pathway remain points of discussion.

The purposes of this study were to determine whether expression of a Bacillus amyloliquefaciens SacB gene in the vacuole or cytosol of maize endosperm would result in accumulation of high-molecular-weight fructan and to determine what effect diverting Sus into this new pathway would have on carbohydrate import, starch biosynthesis, and endosperm development.

MATERIALS AND METHODS

Construction of Endosperm-Specific SacB Expression Vectors

Vacuole-targeted expression vectors were constructed by fusing plant secretion and vacuole-targeting sequences to the mature Bacillus amyloliquefaciens SacB gene. The SacB gene was obtained from the vector pBE311 (Nagarajan and Borchert, 1991). Signal sequences added to the bacterial gene were derived from the sweet potato sporamin (Matsuoka et al., 1990) or barley lectin (Bednarék et al., 1990) genes.

The tissue- and development-specific 10-kD zein promoter (Kirihera et al., 1988) was used for expression of the SacB gene. The promoter and a CaMV 3’ end (Pietrzak et al., 1986) were ligated to the SacB coding regions, using unique restriction enzyme sites included in PCR primers. The vector containing the sporamin peptides was designated pSPor-Sac, and the barley lectin vector was designated pBL-Sac. The completed expression vectors were used separately for direct transformation into maize (Zea mays L.) callus by particle bombardment.

An untargeted SacB expression vector, containing the 10-kD zein promoter and the CaMV 3’ end, was also constructed. The completed plasmid, pCyt-SacB, was used for direct transformation into maize callus by particle bombardment.

Plant Material and Transformation

Plant expression vectors and selectable markers were co-transformed into embryogenic corn callus derived from crosses of the inbred lines A188 and B73 by microprojectile bombardment (Klein et al., 1987). Transformed embryogenic cells were recovered on medium containing either glufosinate-ammonium or chlorsulfuron. The selectable marker pDetric contains the BAR gene, coding for phosphinothricin acetyltransferase, under the control of the 35S promoter. A mutant acetolactate synthase gene contained in pALSLUC confers resistance to chlorsulfuron. Expression of the mutant acetolactate synthase gene is regulated by the CaMV 35S promoter.

Transgenic shoots were transferred to 12-inch pots containing Metromix (Scotts, Marysville, OH) soil and grown to maturity in the greenhouse. Mature R1 seed from original transformants were grown in the greenhouse or planted directly in the field.

Carbohydrate Isolation and Analysis

Bacillus subtilis (BG4103) containing the SacB vector pBE3114 (Nagarajan and Borchert, 1991) was tested for FTF activity by incubation at 37°C for 12 h in Luria broth medium (Sambrook et al., 1989) containing 2% Suc. Briefly, supernatant from the overnight culture was assayed for fructan production by spotting 5 μL directly onto a silica TLC plate (Fisher Scientific). The TLC plate was developed three times in propanol: butanol: water (12:3:4). Fructan, remaining at the plate origin, was detected by staining with a urea-phosphoric acid spray (Wise et al., 1955). Fructan synthesized from the Bacillus line containing a pBE3114 vector was isolated by precipitation with 2 volumes of ethanol. Soluble carbohydrates were separated from fructan by extraction with 80% ethanol at 80°C. Bacterial fructan, described above (20 mg/mL), and Fru (100 mM) were suspended in water and used as positive controls. Glc (100 mM) was used as a negative control.

Transgenic seeds were harvested for carbohydrate analysis at maturity (50–55 DPP) and dried to approximately 12% moisture. Dent and transgenic seeds containing only the selectable marker (pDetric or pALSLUC) were used as negative control samples. Extracts were prepared by homogenizing single seeds soaked in 2 mL of water for 12 to 14 h at 50°C. Extracts were spotted directly onto TLC plates (5 μL) and treated as described above. Hydrolysis of fructan was performed by treating seed extracts with 300 mM HCl at 50°C for 10 min before spotting onto a TLC plate.

Quantitative, single-seed analysis of fructan was performed by a resorcinol method (Roe et al., 1949; Yaphé and Arsenalt, 1965). Briefly, single seeds from independent transgenic lines were incubated for 12 to 14 h in 80% ethanol at 50°C and thoroughly homogenized. The suspension was incubated for 10 min at 80°C, and then centrifuged at 12,000g for 5 min. Ethanol extraction of soluble carbohydrates was repeated two times. The ethanol-insoluble pellets were suspended in 5 mL of water, heated to 80°C for 10 min, and centrifuged for 5 min at 12,000g. The supernatant (35–70 μL) was added to 250 μL of 0.1% resorcinol in ethanol and 1 mL of 30% HCl. The solution was incubated at 80°C for 10 min, which was sufficient for complete hydrolysis of fructan (determined by TLC analysis). Fru was quantified by reading the A330. Glc (100 mM) and dent seed extracts were included as negative controls.

Soluble sugar (Glc, Fru, and Suc) concentration was determined by enzymatic analysis (Brown and Huber, 1987). Prior to analysis, ethanol supernatants (described above) were lyophilized and the carbohydrates were dissolved in water.
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RESULTS

Construction of SacB Expression Vectors

Three expression vectors were constructed containing the B. amyloliquefaciens SacB gene directed by a 10-kD zein seed storage promoter (Kirihara et al., 1988); these are represented in Figure 1. Targeting the bacterial protein to vacuoles in maize endosperm was accomplished by transformation with expression vectors containing secretion and targeting sequences fused to the mature SacB coding sequence. The secretion and targeting sequences were derived from the sweet potato sporamin gene (Matsuoka and Nakamura, 1991) or from the barley lectin gene (Lerner and Raikhel, 1989). The expression vector pCyt-Sac (Fig. 1) was constructed to study the effect of Suc metabolism by the SacB protein in the cytosol of maize endosperm.

Recovery of Transgenic Maize Lines Containing a Vacuole-Targeted SacB Gene

The pSpor-Sac and pBL-Sac expression vectors (Fig. 1) were used to transform maize callus by particle bombardment, and 10 to 15 independent transgenic lines were recovered for each vector. Most lines contained two to three intact copies of the expression cassette and one or more rearranged copies (data not shown). Transgenic shoots or seeds were grown to maturity in the greenhouse or planted directly in the field. Transgenic seeds containing a vacuole-targeted SacB expression vector did not differ phenotypically from transgenic control seeds containing only the selectable marker (pDetric). Transgenic seeds germinated and plants grew to maturity with no detectable differences compared to control lines.

Analysis of Transgenic Seeds by TLC

Figure 2 shows TLC analysis of seed extracts from transgenic lines containing a vacuole-targeted SacB expression vector. Figure 1. Structure of vacuolar and cytosolic expression vectors. Expression of the SacB gene was directed by the 10-kD zein promoter. The vacuolar expression cassette pSpor-Sac contains an N-terminal fusion of the signal peptide and targeting sequence from the sporamin gene. The vector pBL-Sac contains the N-terminal signal peptide and C-terminal vacuole-targeting sequences from a barley lectin gene. The construct pCyt-Sac is an untargeted SacB expression vector, also directed by the 10-kD zein promoter. The CaMV 35S transcription termination region is contained in each construct.

Figure 2. TLC analysis of fructan in transgenic maize kernels. Lanes 1 to 3, Seed extracts from germinated kernels containing the pSpor-Sac construct. Lanes 4 to 6, Extracts from mature pSpor-Sac seeds treated with 300 mM HCl at 50°C. Lane 7, Bacterial levan, treated with HCl at 50°C. Lane 8, Bacterial levan, untreated. Lanes 9 to 13, Single-seed extracts from mature primary transformants containing the pBL-Sac vector. Lanes 14 to 18, Single-seed extracts from mature primary transformants containing the pSpor-Sac vector. Lanes 19 and 20, Untransformed dent seed extracts. Lane 21, Fru control. Lane 22, Glc control.
300 mM hydrochloric acid at 50°C for 10 min resulted in complete hydrolysis of the polymer (Fig. 2, lanes 4–7). Migration of the signal below the solvent front after acid treatment shows that the bacterial polymer and the polymer accumulated in transgenic seed consisted primarily of polymerized Fru.

To study the stability of fructan in maize endosperm during development and germination, the seed coat remaining after transgenic kernels were germinated on agar medium was homogenized and spotted on a TLC plate. Figure 2, lanes 1 to 3, demonstrates that the polymer remained at the origin and was not degraded. This suggests that maize cannot completely remobilize polymerized Fru for the metabolic needs of an emerging seedling. Stability of the Fru polymer during development and germination is not unexpected, since maize does not normally synthesize fructan and would not be expected to express a fructan hydrolase.

Quantitative Analysis of Fructan in Transgenic Seeds

To determine the level of fructan accumulation in seeds containing a vacuole-targeted SacB gene, extracts from 7 to 10 mature kernels containing fructan were assayed by reaction with resorcinol. Figure 3 shows that the average fructan concentration does not vary significantly within a population of seed from the same line or between independent transgenic lines. Fructan accumulation was also relatively constant in seeds from successive generations of the same line and in seeds homozygous or heterozygous for the SacB gene. Concentrations of soluble sugars (Suc, Glc, and Fru) varied among populations of seeds of the same line. However, no significant variation of soluble sugar concentration was found in kernels containing fructan compared to those of controls (data not shown). The data in Figure 3 also demonstrate that the Glc control does not react with resorcinol and that wild-type maize kernels do not contain fructan.

Fructan accumulation was also shown to be similar in mature seeds containing either the barley lectin-SacB gene or the sporamin-SacB gene (Fig. 3). Fructan concentrations in hybrid seed (250x626) derived from crossing a line homozygous for the sporamin-targeted SacB gene (250.73H) with a line homozygous for the barley lectin-targeted SacB gene (626.22H) was no higher than those in the two original parent lines (Fig. 3).

**Accumulation of Fructan in Maize Starch Mutants**

To determine whether the increased Suc that accumulates in a mutant maize line can be utilized for increased fructan synthesis, we crossed representative dent lines containing a vacuole-targeted SacB gene with the sweet corn lines sh1, bt1, and sh2. The hybrid lines were backcrossed to produce F2 seed containing at least one copy of the SacB gene and homozygous for the recessive mutation. The results in Figure 4 demonstrate that the average fructan concentration was significantly higher in each homozygous mutant background compared to dent seeds containing the SacB gene. Suc concentration is known to differ among

![Figure 3](https://www.plantphysiol.org)  
**Figure 3.** Fructan concentration in transgenic dent seed extracts by resorcinol analysis. Fructan concentration was determined in extracts prepared from 7 to 10 mature seeds containing the pBL-Sac and pSpor-Sac constructs. Independent primary transformants containing the pBL-Sac vector are designated 626.1 and 625.1. The line 626.132 represents seed derived from selfing the primary transformant 626.1. Primary transformants containing the pSpor-Sac vector are designated 4085 and 250.1. The line designated 250.73 represents seeds derived from crossing the primary transformant 250.1 with a dent maize line. Seeds from the lines 626.22H and 250.73H did not segregate on the ear for the SacB vector and were confirmed to be homozygous for the SacB gene by outcrossing with dent lines. The line 250x626 represents mature seed resulting from a cross between lines 626.22H and 250.73H, using 626.22H as the male. The line 626x250 represents mature seed resulting from the cross between lines 250.73H and 626.22H, with 250.73H as the male. Glc and untransformed dent seeds did not react with resorcinol.

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250
200
150
100
50

Figure 4. Fructan concentration in mutant maize seed containing a vacuole-targeted SacB vector. Fructan concentration from 7 to 10 mature mutant seeds was determined by resorcinol analysis. Kernels were determined to be homozygous for the mutant allele by kernel phenotype. The lines 626-sh2, 626-bt2, and 626-sh1 are heterozygous for the pBL-Sac vector and homozygous for the sh2, bt2, and sh1 alleles, respectively. The lines 4085-sh2 and 226-sh2 are heterozygous for the pSpor-Sac vector and homozygous for the sh2 allele. The transgenic dent lines 626.1 and 202.10 contain the pBL-Sac and pSpor-Sac vectors, respectively.

these mutants (reviewed by Doehlert and Kuo, 1994; Nelson and Pan, 1995), and fructan accumulation was shown to reflect the level of Suc. Fructan concentration was lowest in dent seeds, accounting for 1% of the dry weight, and approximately 2-fold higher in the sh1 background. Fructan accumulation in sh2 and bt2 mutants, which accumulate the highest levels of Suc, averaged from 4 to 8% of the dry weight of mature seeds. The highest level of fructan accumulation in a sh2 background was approximately 8 to 9 times higher than that found in dent seeds.

Dry-Matter Accumulation in Mature Transgenic Seeds

To study the effect of fructan synthesis on dry-matter accumulation in mature seeds, the dry weight of 10 to 15 seeds containing fructan was compared to that of an equal number of negative controls (seed segregating on the same ear that did not contain a vacuole-targeted SacB gene). Figure 5A demonstrates that the accumulation of fructan in transgenic seeds did not significantly increase the mature dry weight, compared to dent or negative control seeds. Figure 5B also shows that no significant increase in seed weight was found in homozygous mutant kernels containing a vacuole-targeted SacB gene compared to shrunken seeds that did not accumulate fructan. Increased Suc metabolism and fructan accumulation in a mutant kernel containing the SacB gene did not restore the wild-type phenotype.

Cytosolic Expression of the SacB Gene in Maize Endosperm

To determine the effects of cytosolic expression of the SacB gene on starch biosynthesis and endosperm development, maize callus was transformed with an untargeted SacB expression vector (pCyt-Sac). Transgenic shoots, grown to maturity under greenhouse conditions, produced R1 seed segregating for the cytosolic expression vector. Kernels containing the cytosolic expression vector could easily be distinguished from control seed (without the pCyt-Sac vector) by their color (Fig. 6A). Although the appearance of kernels containing pCyt-Sac was dramati-
Figure 6. Cytosolic expression of SacB in dent maize endosperm. Independent transgenic maize lines were recovered containing at least one copy of the pCyt-Sac vector. A, Seeds from a primary transformant segregating for the pCyt-Sac vector. Seeds containing the cytosolic vector appear darker than negative controls at 30 to 35 DPP. B, Mature seeds from the pCyt-Sac line 654.2 (right) compared to seeds containing the pSpor-Sac gene. C, Dry weight comparison of mature seeds containing the pCyt-Sac vector compared to negative controls and nontransformed dent seeds. The hatched bars represent seeds containing a SacB gene, and the solid bars are negative controls. D, Thin sections of mature seeds containing the pCyt-Sac vector (left) compared to mature pSpor-Sac seeds (right) each stained with an iodine solution. Dark staining indicates the presence of starch.

cally altered, seed size was not a distinguishing factor early in development and was only slightly different at 30 to 35 DPP (Fig. 6A). During later stages of development, when seeds were drying, transgenic kernels could be differentiated by a significant reduction in their size. Mature dry seeds containing the cytosolic sacB were much smaller than were mature dry seeds containing a vacuole-targeted SacB gene (Fig. 6B). The dry weight was shown to be 10-fold lower for transgenic seeds containing the pCyt-Sac vector compared than for control or dent seeds (Fig. 6C). The results indicate that during development, when dent kernels accumulate starch, transgenic seeds contain-
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Equivalent levels of fructan accumulation were demonstrated in several independent transformed lines and in lines homozygous for either the barley lectin or sporamin-targeted SacB gene (Fig. 3). Fructan accumulation was not significantly different in kernels containing one, two, or three endosperm copies of the SacB gene. A dose-response relationship between gene copy number and the level of fructan could not be established, which suggests that expression of the vacuole-targeted SacB gene in endosperm is not the limiting factor in fructan synthesis.

Fructan accumulation in seeds did not result in a significant increase in dry-matter accumulation (Fig. 5, A and B). This result indicates that the vacuolar Suc pool may be isolated from the cytosolic pool and that exchange between the two compartments is limited. Import of Suc into the vacuole by a relatively slow, passive process could explain the limited exchange of Suc between the cytosol and vacuole. Slow diffusion of Suc from the cytosol by a passive process is consistent with the suggestion that the vacuolar Suc concentration limits fructan synthesis and could explain why fructan synthesis in transgenic seeds containing the SacB gene did not increase overall dry-matter accumulation. These results are also consistent with the study by Felker et al. (1990), who suggest that the vacuole in dent endosperm cells plays a very minor role as a storage site for Suc and other sugars.

Vacuole Expression of SacB in Mutant Maize Endosperm

If Suc concentration in the vacuole of dent endosperm limits fructan synthesis, then transfer of the targeted SacB gene into a high-Suc mutant line might allow higher levels of fructan accumulation. Increased Suc concentration has been reported in maize starch mutants (Creech 1965; Han- nah et al., 1980; Bhave et al., 1990) and varies depending on the specific mutation (Doehlert and Kuo, 1994).

Mutant lines containing a vacuole-targeted SacB gene accumulated significantly higher levels of fructan compared to dent kernels containing the same gene (Fig. 4). The increased Suc concentration in mutant endosperm cells appeared to equilibrate with the vacuole and was available for fructan synthesis. The level of fructan accumulated reflected the difference in Suc concentration in each distinct mutant. The results also suggest that the concentration of vacuolar Suc affects the level of fructan synthesis.

Cytosolic Expression of SacB in Maize Endosperm

Targeting the SacB protein to maize endosperm vacuoles resulted in fructan accumulation in a kernel that was otherwise indistinguishable from wild-type seeds. In contrast, cytosolic expression of the SacB gene was devastating to endosperm development. Early in development the kernel size gave no indication of reduced dry-matter accumulation (Fig. 6A), but the dry weight of mature kernels accumulating cytosolic fructan was reduced to approximately 10% of that of mature dent seeds (Fig. 6C). Fructan accumulation was relatively low, approximately 1.6% of the dry weight, in mature seeds containing the pCyt-Sac gene. The most significant effect of diverting cytosolic Suc into the

DISCUSSION

This study demonstrates that expression of the B. amyo- liquefaciens SacB gene in maize endosperm results in conversion of Suc to a high-molecular-weight Fru polymer. Stable accumulation of the polymer in seeds is presumed to be due to the absence of a fructan-specific hydrolase in maize. Fructan accumulation in seeds containing a vacuole-targeted SacB gene did not alter endosperm development, dry matter accumulation, or seed germination. However, expression of SacB in the cytosol of maize endosperm caused a dramatic reduction in starch synthesis, mature seed dry weight, and seed germination. Contrasting phenotypes demonstrated in transgenic maize lines containing the vacuole-targeted or cytosolic SacB gene emphasize the role of Suc and Suc partitioning in starch synthesis and endosperm development.

Vacuole Expression of SacB in Dent Maize Endosperm

Detection of SacB protein in transgenic tissue containing the vacuole-targeted gene with SacB-specific antisera was not successful. The level of protein in transgenic tissue proved to be well below the limit of detection. However, qualitative and quantitative detection of fructan confirmed that SacB was present and active in transgenic maize endosperm. This result is similar to those in previous reports describing expression of a bacterial SacB gene in transgenic plants. The chimeric Bacillus protein could not be detected in extracts prepared from either transgenic tobacco or potato plants (Ebskamp et al., 1994; van der Meer et al., 1994).

In contrast to the report of vacuole-targeted expression of a SacB gene in potato microtubers (Ebskamp et al., 1994), development of maize endosperm expressing a vacuole-targeted SacB gene was not significantly different compared to controls. No detectable differences in the levels of soluble carbohydrates or dry-matter accumulation were found in seeds accumulating fructan compared to negative controls or dent seeds. Conversion of the vacuolar Suc pool into hexose and fructan did not alter mature seed dry weight, seed morphology, or germination. The results suggest that Sac contained in the vacuole is not critical to maize endosperm development or to seed germination.

ing the cytosolic SacB expression vector accumulate mostly water. Mature pCyt-Sac seed also germinated at very low rates (less than 3% germination compared to 85% germination of pSpor-Sac seeds) under greenhouse conditions.

Thin sections of mature seeds containing either the pCyt-Sac or pSpor-Sac vector differed when treated with an iodine solution (I₂/KI). Kernels containing the pSpor-Sac vector stained dark blue, demonstrating the presence of starch, but seed containing the cytosolic SacB gene did not stain, suggesting a severe reduction in starch synthesis (Fig. 6D). Only an occasional starch granule could be found in seeds containing the pCyt-Sac vector when viewed under polarized light (data not shown). Fructan levels in mature dry seeds containing the pCyt-Sac vector averaged 16 to 18 mg/g.

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new fructan pathway was on accumulation of starch in the endosperm (Fig. 6D).

**Cytosolic Expression of SacB and Maize Starch Biosynthesis**

The precise route for conversion of Suc to starch in maize is not yet clear. High levels of invertase activity localized at the base of a kernel have been demonstrated (Shannon, 1972), and the resulting hexose may be phosphorylated and enter the starch pathway independently of SS (Dohlert et al., 1988; Felker et al., 1990). However, it has also been shown that hydrolysis of Suc is not an absolute requirement for movement of Suc into the endosperm (Schmalstig and Hitz, 1987). A significant amount of Suc may be directly converted to Fru and UDP-Glc by SS and then utilized for starch synthesis in the upper endosperm.

Glc is an end product of the Fru polymerization reaction and the SacB protein has been shown to act exclusively as an invertase in Suc concentrations below 50 mM, releasing Glc and Fru (Chambert and Petit-Glatron, 1993). In kernels containing the pCyt-Suc gene, hexose was not converted into a significant level of starch, which is consistent with the hypothesis that UDP-Glc, and not hexose, is the critical substrate for starch biosynthesis in maize endosperm.

Analysis of sh1 mutant maize lines has been invaluable in determining the significance of SS in the biosynthesis of starch. The SS2 protein, coded for by the Sus gene, was found to be active in maize endosperm when it was discovered that deletion of the sh1 gene did not result in a complete loss of SS activity or starch synthesis (McCarty et al., 1986; Chen and Chourey, 1989). Recently, a double mutant at both the Sh and Sus alleles was described that accumulates starch at approximately the same levels as the single sh mutant (Chourey and Taliercio, 1994). Disruption of both known SS genes without significant loss of starch could indicate that a significant amount of Suc is converted to starch by a route other than through SS. However, SS activity is not completely abolished in the double mutant (P.S. Chourey, personal communication) and it is possible that very low levels of SS activity are sufficient for moderate levels of starch synthesis. Zrenner et al. (1995) demonstrated that reduction of SS activity in transgenic potato tubers by 96% resulted in starch accumulation at approximately 34% of the rate in wild type. Starch synthesis was reduced, but not abolished, in the transgenic tubers, whereas hexose concentration was found to be increased by approximately 200-fold relative to wild-type controls.

The results demonstrate that UDP-Glc, not hexose, is critical to the synthesis of starch in potato. The present study demonstrates that hexose is not utilized for starch synthesis in maize, which is consistent with studies in potato as well as in wheat and barley (Morrell and ap Rees, 1986; Hawker et al., 1991; Duffus and Cochraine, 1992). Together, these studies suggest that invertase does not play a major role in conversion of Suc to starch.

One other possible explanation for the reduction of starch synthesis, altered osmotic potential in the endosperm due to fructan accumulation, may be ruled out because of the very low levels of fructan in mature kernels. A modest drop in starch synthesis would be expected in proportion to increasing fructan levels, rather than the near complete absence of starch demonstrated in this study. It seems more likely that SS present early in development is less effective in competing for Suc ($K_m = 192$ mm; Chourey, 1981) than the SacB protein ($K_m = 20$ mm; Chambert and Petit-Glatron, 1993). The dramatic phenotype of transgenic seeds containing an untargeted SacB gene emphasizes the crucial role of UDP-Glc, catalyzed by SS, in the maize starch biosynthetic pathway.

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