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W.-H. Cheng · P. S. Chourey

Genetic evidence that invertase-mediated release of hexoses is critical for appropriate carbon partitioning and normal seed development in maize

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Abstract Cell wall-bound invertase (CWI) is spatially and temporally the first enzyme which metabolizes the incoming sucrose in developing seed of maize (*Zea mays*). Our previous studies have shown that the cell wall-bound invertase-2 (INCW2) isozyme encoded by the wild-type gene of the *Miniature1* (*Mn1*) seed locus plays a critical role in seed development. Null mutations of the gene, such as the *mn1* seed mutant which lacks invertase activity, are associated with a loss of ~70–80% of the normal seed weight. We show here that under in vitro kernel culture conditions the hexose-based medium was similar to the sucrose-based medium in promoting the normal development of kernels of the *Mn1*, but not of the mutant *mn1*, genotype. Anatomical, biochemical, and immunohistological data showed that the *mn1* kernels retain their mutant phenotype regardless of the presence of sucrose or hexoses in the culture media. The most drastic changes in the *mn1* seed mutant were associated with a significant reduction in the size of the endosperm, but not in the pattern or the level of starch localization. Because *Mn1* expression was temporally coincident with the endosperm cell divisions, INCW2 must play a critical role in providing hexose sugars for mitotic division, and only a minor role in generating carbon skeletal substrates for starch biosynthesis in the early stages of endosperm development. Furthermore, a lack of the wild-type seed phenotype of the *mn1* mutant in hexose media suggests that a metabolic release of hexoses catalyzed by INCW2, rather than an exogenous source, is critical for both generating appropriate sugar-sensing signals for gene expression and for normal endosperm development.

Key words *Zea mays* · Invertase · *Miniature1* seed mutant · Kernel culture · Futile cycle

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Wan-Hsing Cheng
Program in Plant Molecular and Cellular Biology, Plant Pathology Department, University of Florida, Gainesville, Florida 32611–0680, USA

Prem S. Chourey (✉)
Program in Plant Molecular and Cellular Biology, Departments of Plant Pathology and Agronomy, University of Florida, Gainesville, Florida 32611-0680, USA and U.S. Department of Agriculture, Agricultural Research Service, Gainesville, Florida 32611-0680, USA
Fax: +1(352)-392-6532
E-mail: psch@gnv.ifas.ufl.edu

Introduction

Seed development is highly dependent on the metabolic utilization of sucrose. Sucrose is both the principal and the preferred form of photosynthate for long-distance transport to terminal storage sink tissues such as developing seeds in maize and other cereals. Sucrose also serves as a signal molecule in regulating gene expression and in normal development in diverse cell types in a range of plant species (see the recent reviews of Jang and Sheen 1997; Smeekens and Rook 1997; Weber et al. 1997 b), in addition to being a sole source of carbon for numerous metabolic processes and storage as starch. Despite such a global role of sucrose in metabolic and developmental biology, there are large gaps in our knowledge on the specific genes and physiological processes that may be critical to the unloading and utilization of sucrose in economically important tissues such as developing seeds.

In maize, several lines of evidence indicate that a substantial proportion of photo-assimilated sucrose, probably >70% of the total transported to the pedicel through phloem termini, is unloaded into a developing endosperm (Hanft and Jones 1986; Schmalstig and Hitz 1987; Felker 1992; Miller and Chourey 1992; Shannon et al. 1993). Once inside the endosperm, the *Mn1*-encoded cell wall-bound invertase-2 (INCW2) constitutes the entry point for the sucrose carbon into

numerous metabolic reactions. Indeed, the loss of INCW2 is the causal basis of the *mn1* seed phenotype, marked by a coniderable reduction in the flux of sucrose into subsequent metabolic reactions in a developing seed and a loss of about 70–80% of mature seed weight (Cheng et al. 1996). The invertase-deficiency in the *mn1* endosperm cells is also postulated to cause cell degeneration in placento-chalazal cells of the pedicel (Miller and Chourey 1992), leading to a premature gap formation between the pedicel and the endosperm, first described by Lowe and Nelson (1946). These genetic data clearly indicate that cell-wall invertase in an endosperm is critical for the appropriate partitioning of sucrose in a developing seed. A similar role of cell-wall invertase in the early metabolic utilization of sucrose is also implicated in the developing seeds of *Vicia faba* (Weber et al. 1996 a).

It is, however, unclear as to how sucrose is unloaded or transferred from the pedicel to the endosperm which is symplastically discontinuous from the maternal cells at the base of the seed. Based on biochemical genetic analyses on the *mn1* seed mutation, we have suggested, among other possibilities, that invertase-mediated hydrolysis of sucrose in basal endosperm cells may exert a regulatory force in the pull of the photosynthate column from the pedicel and into the endosperm (Miller and Chourey 1992). This was in agreement with previous suggestions that sugar uptake is a passive process (Porter et al. 1985; Schmalstig and Hitz 1987) and that uptake might be regulated by metabolic processes within the endosperm (Griffith et al. 1987). These data do not rule out the possibility of a membrane-associated carrier/transporter as postulated by Felker (1992) in maize, and demonstrated in *V. faba* developing seeds (Weber et al. 1997 a). However, our efforts to identify such a protein or a gene in maize have so far been unsuccessful.

Gengenbach (1977) first described the method of in vitro culture of maize kernels. This allows the in vitro development of kernels, as early as 0 days after pollination (DAP), in a well-defined medium giving rise to physiologically and anatomically mature kernels similar to those grown *in planta*. The method has been utilized extensively to analyze the effects of the environment, and of diverse sources of carbohydrates or other metabolites on kernel growth and development (Shimamoto and Nelson 1981; Cobb and Hannah 1983; Hanft and Jones 1986; Griffith et al. 1987; Felker 1992). Furthermore, it is significant that the in vitro culture medium supports normal kernel growth on either sucrose or hexoses as a carbon source (Cobb and Hannah 1986, 1988; Griffith et al. 1987). Thus, we reasoned that the *mn1* seed mutant might be restored to a complete, or near complete, normal seed phenotype by in vitro culture of the mutant kernels in media with reducing sugars. It was expected that kernel growth on hexose-based media would bypass the bottleneck caused by the invertase-deficiency of the *mn1* seed mutation. Remarkably, the data from the present study show that the use of hexose sugars failed to restore the normal seed phenotype (i.e., the phenocopies) of the *mn1* seed mutant; yet the same hexose-based medium produced normal kernels with the *Mn1* genotype. We believe these observations constitute the first genetic evidence that the metabolic release of hexoses is critical for the normal partitioning of sucrose in a developing endosperm.

Materials and methods

Plant materials

The homozygous *Mn1Mn1* maize plants used in this study were either Wisconsin 22 (W22) or Pioneer 3165 inbred lines; the *miniature1* (*mn1*) seed mutant was in a W22 genetic background. All plants were grown in the greenhouse or field; the temperature was controlled between 28 and 32°C, in the former with a normal diurnal (day/night) pattern. Young ears at 3 DAP were harvested and immediately brought to the laboratory for subsequent kernel culture under aseptic conditions.

Kernel culture and growth conditions

After removing about four layers of outer husks, young ears were sterilized with a 95% alcohol-spray and dried for 2 min under the laminar flow hood. Then, the inner husks and silks were removed using forceps. In a moist dish, a scalpel was used to longitudinally dissect the ear into two-row pieces (Fig. 1) with a total of six-attached kernels, each row with three kernels. After further severing most of the white pith tissue and removing three kernels from alternating rows of block, the blocks with three-attached kernels were placed on 100 × 25-mm plastic Petri dishes, each plate with four blocks, containing various sugar-supplemented media, and incubated in a dark growth chamber at 28°C. The preparation of media was essentially as described by Gengenbach and Green (1975). The media were supplemented with 1.0 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 g/l of casein hydrolysate (Cobb and Hannah 1986). All media were adjusted to pH 5.8 prior to the addition of agar (5.5 g/l). After autoclaving, the media were cooled to 50°C and filter-sterilized streptomycin sulfate (10 mg/l) and 15% sugar (sucrose, fructose, glucose, or fructose + glucose) were added. Culture medium with extremely high levels of sugars (15%) and phytohormone, 2,4-D is designed to maintain an *in planta* environment for kernel growth. After 14–16 days of culture, the blocks were rinsed with distilled water to remove residual components of the medium and drained on Kimwipes for about 2 min. Subsequently, kernels with pedicels and cob tissues were separated with a scalpel; some of them were immediately fixed in formalin acetic alcohol (FAA) for anatomical and immunohistochemical analyses, as described below, and the remainder were frozen by adding liquid N_2, followed by storage at −80°C until further use.

Anatomical analyses and immunohistochemical localization of cell wall-bound invertase

Developing kernels *in planta* or in vitro at various stages were harvested and immediately fixed in formalin acetic alcohol (FAA), dehydrated through a tertiary butyl alcohol (TBA) series, infiltrated in Paraplast plus paraffin (Fisher Scientific), embedded, and immunostained essentially following the protocol described previously

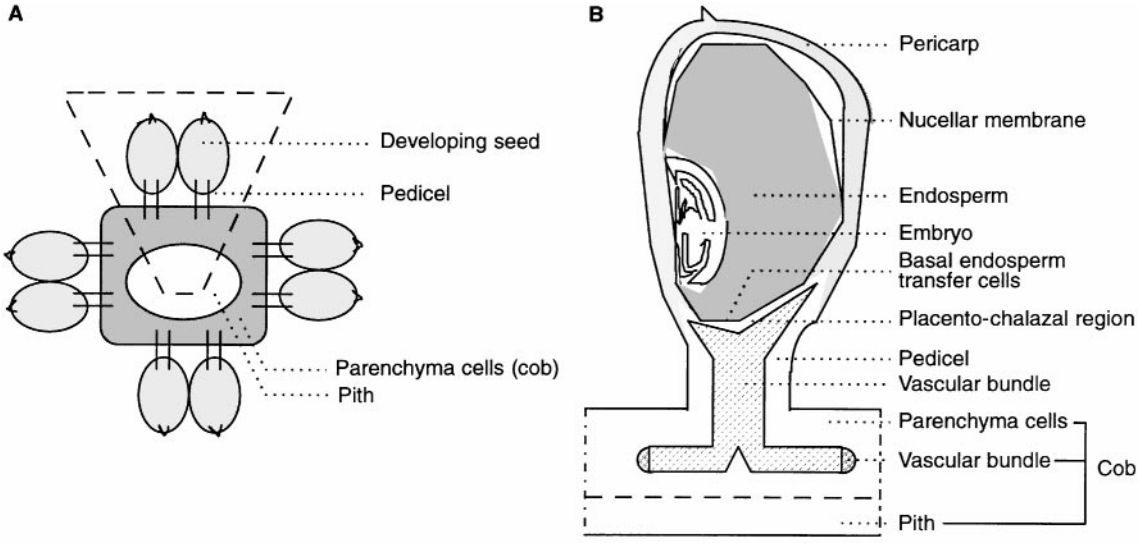

Fig. 1A, B Schematic diagram of developing seeds of maize. **A** Cross section of a young ear showing the paired arrangement of kernels on the cob. The *dashed sector* represents the paired row blocks dissected for kernel culture. **B** Longitudinal section of a developing kernel attached to the cob. The scale in this diagram does not reflect the exact size of kernels *in planta*

(Chen and Chourey 1989; Cheng et al. 1996). Polyclonal antibodies of cell wall-bound invertase were raised in a rabbit against carrot cell wall-bound invertase protein. This antibody was a gift from Dr. Arnd Sturm (FMI, Basel, Switzerland). After primary antibody treatment and subsequent washing, the slides were then incubated with a secondary antibody solution composed of biotinylated anti-mouse anti-rabbit immunoglobulin and streptavidin alkaline phosphatase. The immunolocalized signal of cell wall-bound invertase was visualized using New Fuchsia chromogen (Dako Corporation, Carpinteria, Calif.), resulting in a precipitate of fuchsia-colored end product at the site of the antigen.

Invertase enzyme assays

Frozen kernels were homogenized in extraction buffer in a 1:10 (w/v) ratio using a chilled mortar and pestle. The extraction buffer employed in the isolation of soluble invertase protein contained 50 mM of Tris-maleate, pH 7.0, and 1 mM of DTT (Doehlert and Felker 1987). The homogenate was centrifuged at 14 000 g for 10 min; the supernatant was retained and dialyzed overnight prior to soluble invertase assays. The pellet containing cell wall-bound invertase was washed three times by re-suspending in extraction buffer and centrifuged at 14 000 g for 10 min. The subsequent pellet was re-suspended in extraction buffer containing 1 M NaCl in a 1:2 (w/v) ratio, shaken on a rotating shaker for about 2–3 h at 3°C, and subsequently centrifuged at 14 000 g for 10 min. The final supernatant, enriched for cell wall-bound invertase, was dialyzed against extraction buffer without NaCl at 3°C overnight and assayed for enzymatic activity, as described previously (Tsai et al. 1970; Miller and Chourey 1992). The enzyme reaction buffer was composed of 0.2 M sodium acetate (pH 4.8) and 50 µl of 0.04 M sucrose as a substrate. Crude protein extract was added to the reaction buffer for a total reaction volume of 200 µl, followed by incubating in a 37°C water bath for 15 min. The reaction products were determined by Nelson's test (Nelson 1944).

Protein gel blot analysis

Denatured protein samples obtained from an undialyzed soluble, or a dialyzed cell wall-bound form, of crude extract were separated on an SDS-polyacrylamide gel. The blotting, immunostaining, and further protein visualization were described previously (Cheng et al. 1996). Primary antibodies included polyclonal CWI, the ADP-glucose pyrophosphorylase (ADPGase) small subunit encoded by *Bt2*, and monoclonal sucrose synthases SS1 and SS2 encoded by *Sh1* and *Sus1*, respectively (Chourey et al. 1991 b). ADPGase antibody was a gift from Dr. L. Curtis Hannah and characterized previously by Giroux and Hannah (1994).

Results

Seed phenotypes of kernels cultured in vitro

Figure 2 represents the seed phenotypes of kernels cultured for 14 days on various sugar-supplemented media. The homozygous *Mn1* kernels retained a normal wild-type seed phenotype, regardless of the source of carbon sucrose, fructose, glucose, or fructose + glucose. Figures 2 A and B represent kernels grown on sucrose-supplemented medium; the kernel size was similar to, or only slightly smaller than, that *in planta* at 14 DAP. Similarly, the homozygous *mn1* kernels showed no change in seed phenotype as compared to the kernels grown *in planta*, regardless of the presence of sucrose, fructose, glucose, or fructose + glucose. Figures 2 C and D depict a sample of homozygous *mn1* kernels grown on fructose. Typical features of the *mn1* seed mutant phenotype, such as miniature seed size and papery pericarp, were also seen when these kernels were cultured on the same sugars as mentioned above.

To eliminate possible genotypic effects of maternal tissues (pedicel and cob) on the absorption of nutrients from the medium and subsequent transport into the endosperm, we grew the progeny of self-pollinated heterozygous *Mn1mn1* kernels on various

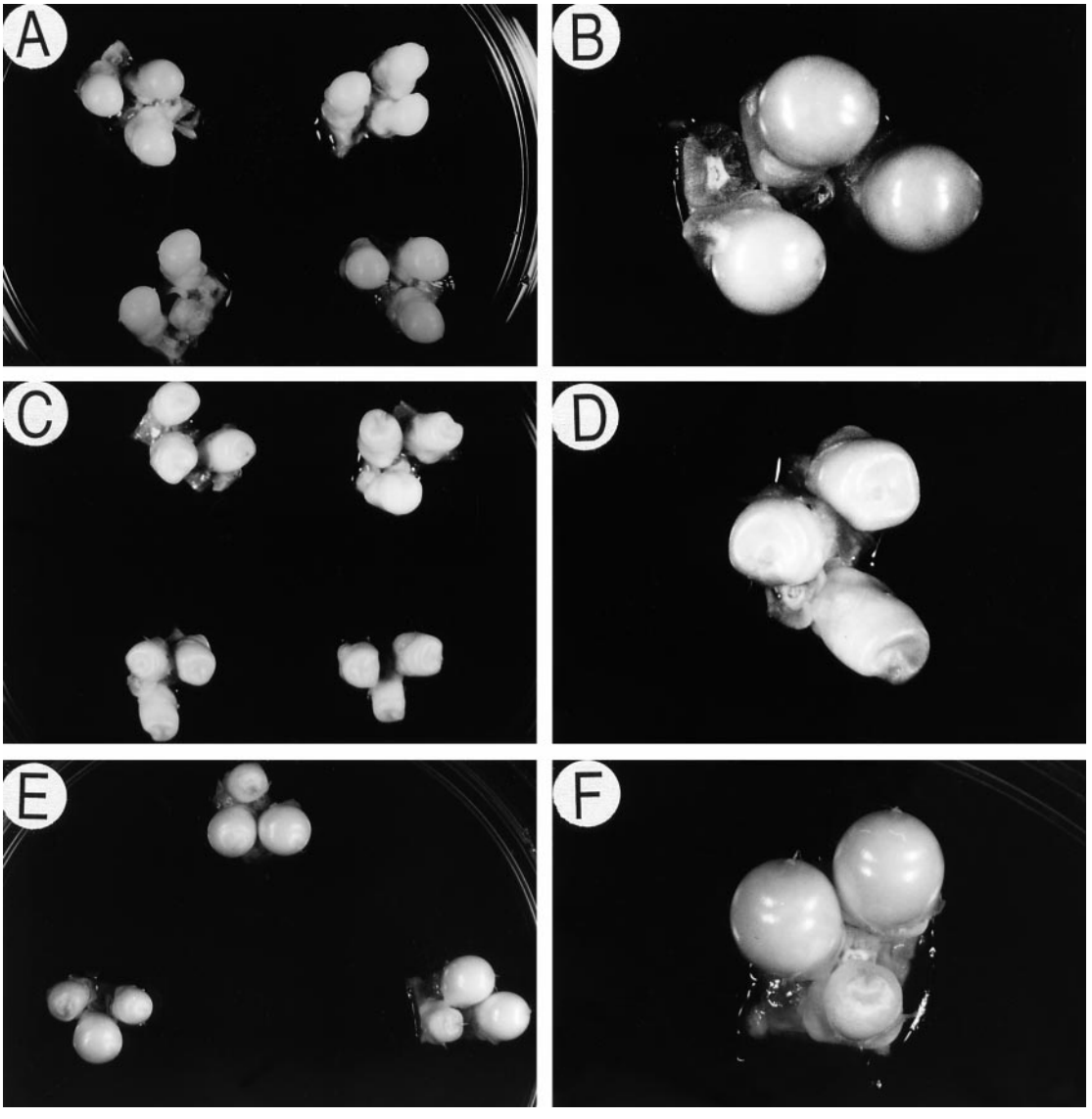

Fig. 2A–F Seed phenotypes of kernels cultured on various sugars for 14 days. **A** and **C** represent homozygous *Mn1* and *mn1* kernels cultured on sucrose- and fructose-supplemented medium, respectively. **B** and **D** represent close-up views of one block of explants shown in **A** and **C**, respectively. **E** Self-pollinated heterozygous *Mn1mn1* kernels grown on glucose-supplemented medium showing *mn1* seed segregants among the normal wild-type seeds on the same cob blocks. **F** A close-up view of one block in **E**

sugar-supplemented media. The resulting F_2 hybrid displayed segregation for the miniature seed phenotype in each medium. As shown in Figs. 2 E and F, the *mn1* mutant seeds grown on glucose segregated from the normal wild-type seeds on the same cob along with the *Mn1mn1* genotype. Invertase activity in segregating miniature seeds (data not shown) was similar to the values given in Table 1, whereas the wild-type seeds (*Mn1*/-/-) displayed a lower level of enzyme activity as compared to the homozygous *Mn1* kernels in the corresponding medium (Table 2). Enzyme activity ranged from approximately 20 to 60% of normal wild-type activity *in planta* (data not shown), presumably due to the segregation of homozygous and heterozygous kernels for the *Mn1* allele. The wild-type kernels used for enzyme assay might have mixed genotypes of one, two, or three copies of the *Mn1* locus. As described previously (Cheng et al. 1996), invertase activity in triploid endosperm is gene-dose dependent on the *Mn1* locus. Thus, these data clearly indicate that the *mn1* seed phenotype seen in kernel culture is not due to the effect of maternal tissue because the wild-type and *mn1* mutant seeds were segregating on the same cob tissue of the *Mn1mn1* genotype. These data are in agreement with the *in planta* studies described previously (Miller and Chourey 1992). In-vitro kernels cultured on a medium without sugar showed no growth, except for slightly swollen, translucent and fragile kernels (data not shown), primarily due to carbon source deprivation.

Table 1 Invertase activity of homozygous *mn1* kernels cultured on various sugars for 14 days

Enzyme activity[a] (μmol reducing sugar/mg protein per min)				
Medium	Bound	Soluble	Total	(%)[b]
Planta (*mn1*)	0.029 ± 0.011	0.009 ± 0.002	0.038 ± 0.013	(100)
+Suc	0.032 ± 0.001	0.020 ± 0.006	0.051 ± 0.007	(134)
+Fru	0.023 ± 0.004	0.019 ± 0.004	0.042 ± 0.009	(111)
+Glc	0.033 ± 0.000	0.018 ± 0.000	0.051 ± 0.000	(134)
−Sugar	0.042 ± 0.004	0.038 ± 0.001	0.080 ± 0.003	(211)

[a] Values are means ± SD of two independent experiments, each experiment with two measurements
[b] Values within parentheses refer to *in-planta mn1* kernels

Table 2 Invertase activity of homozygous *Mn1* kernels cultured on various sugars for 14 days

Enzyme activity[a] (μmol reducing sugar/mg protein per min)				
Medium	Bound	Soluble	Total	(%)[b]
Planta	1.022 ± 0.004	0.136 ± 0.003	1.158 ± 0.001	(100)
+Suc	0.796 ± 0.004	0.136 ± 0.001	0.922 ± 0.011	(79.7)
+Fru	0.822 ± 0.037	0.113 ± 0.019	0.935 ± 0.018	(80.7)
+Glc	0.638 ± 0.123	0.137 ± 0.008	0.775 ± 0.115	(66.9)
−Sugar	0.154 ± 0.008	0.045 ± 0.013	0.198 ± 0.006	(17.1)

[a] Values are means ± SD of two independent experiments, each experiment with two measurements
[b] Values within parentheses refer to kernels *in planta*

Anatomical analysis of the wild-type and *mn1* kernels grown *in planta* and in vitro

Longitudinal sections of the *Mn1* and *mn1* kernels from *in planta* growth at 14 DAP and in vitro kernel culture for 14 days were stained with fast green and I_2KI, and then analyzed for cellular details and starch localization, respectively (Fig. 3). In maize, cell division in a developing endosperm is nearly completed by 14 DAP (Kiesselbach 1949). In the *Mn1* kernels at this stage of development, the entire nucellar region was replaced by the endosperm tissue in both *in planta* and in vitro grown kernels (Fig. 3 A and E). In contrast, the endosperm in the *mn1* mutant remained highly diminutive in size, in both *in planta* and *in vitro* kernels (Fig. 3 B and F). Although the aleurone layer of the *Mn1* endosperm was in tight contact with the pericarp, much empty space between the two cell types was readily visible in the *mn1* kernels (Fig. 3 C), forming a papery pericarp appearance. Remarkably, the *mn1* kernels cultured in glucose-based medium have also retained the same anatomical features (Fig. 3 G) as the kernels grown *in planta*. A similar anatomical pattern was also seen in kernels grown on the other sugars (data not shown). The *mn1* kernels cultured on glucose medium exhibited a characteristic feature of the early development of a gap between pedicel and endosperm (Fig. 3 H), similar to that seen *in planta* (Fig. 3 D), and also described previously (Lowe and Nelson 1946; Miller and Chourey 1992; Cheng et al. 1996).

As for the starch deposition in endosperm, it was striking that there was very little difference between the two genotypes, both *in planta* and in vitro grown kernels. Importantly, similar levels and patterns of starch localization were seen among the three sugars tested for the *mn1* kernels (data not shown). A slight increase in the greater density of starch grains in the *Mn1* and *mn1* sections from the *in planta* as compared to the in vitro kernels was presumably due to developmental differences in the two growth environments.

Invertase activities in kernels cultured in vitro

Tables 1 and 2 show invertase activity in crude extracts of 14 DAP *Mn1* and *mn1* kernels, respectively, in vitro cultured on sucrose, glucose or fructose. As shown previously for the *in planta* kernels (Miller and Chourey 1992; Cheng et al. 1996), cell-wall invertase was the predominant form of the enzyme in developing kernels of both genotypes, and the *mn1* kernels exhibited extremely low levels of enzyme activity as compared to the *Mn1* genotype. Although the in vitro *Mn1* kernels have shown about 20% reduction in total enzyme activity as compared to those grown *in planta* (Table 1), it is remarkable that similar levels of enzyme activity were seen in kernels grown on the three different sugars. In particular, the kernels cultured on sucrose- or hexose-based medium have shown no major difference in the levels of total invertase activity in these two genotypes. Previously, Cheng et al. (1996) have observed coordinate control between the cell-wall and soluble forms of invertases in developing kernels of various genotypes. The in vitro kernels from two of the three media failed to show such a control between the two isozymes. For example, there was no change

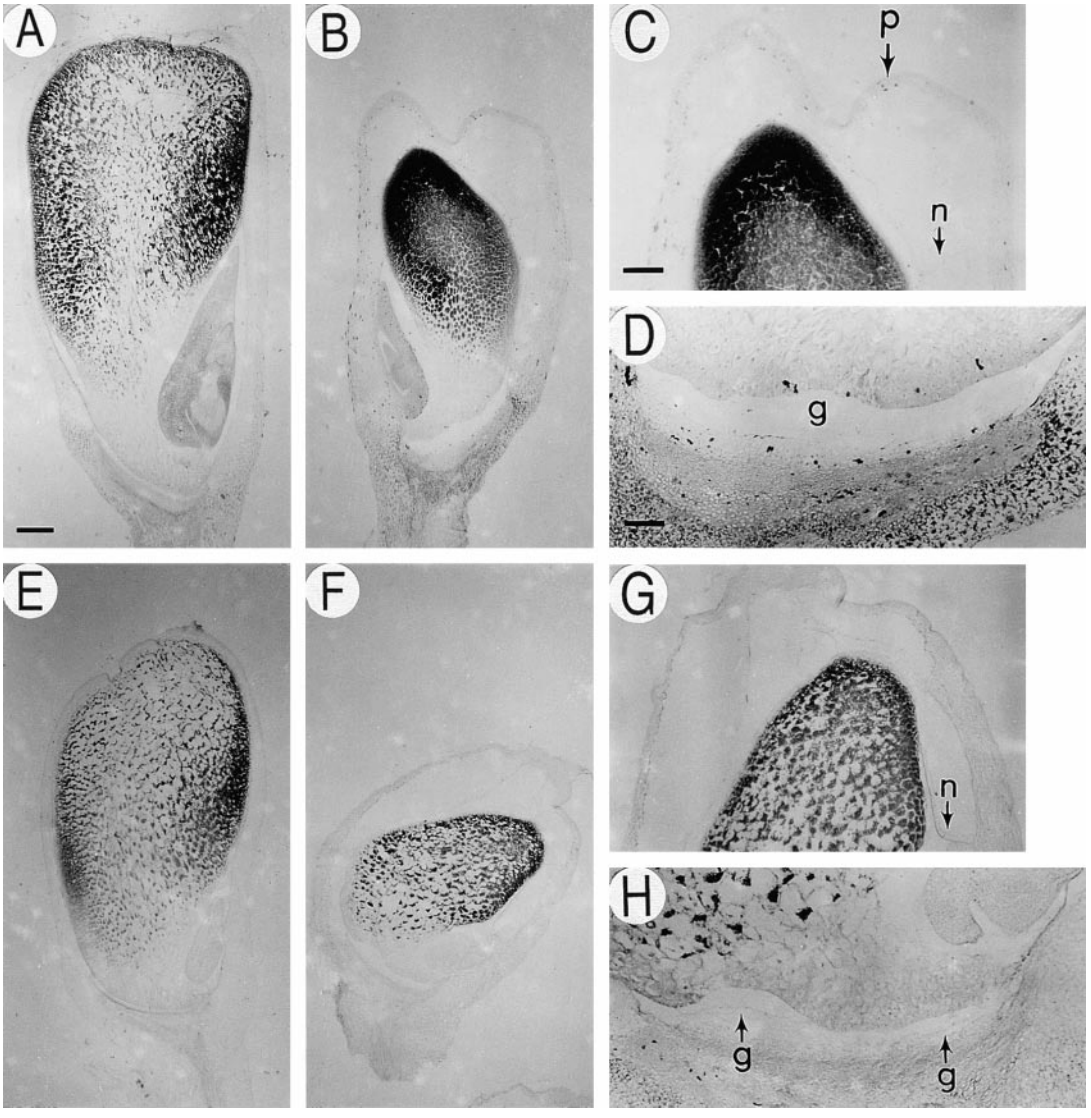

Fig. 3A–H Anatomical comparison between wild-type and the *mn1* mutant kernels, grown *in planta* at 14 DAP and in vitro for 14 days. **A** and **E** represent *Mn1* kernels *in planta* and in vitro (sucrose), respectively. **B** and **F** represent *mn1* kernels *in planta* and in vitro (glucose), respectively. **C** and **D** are close-up views of **B** at the upper and lower parts of the kernel, respectively. **G** and **H** are close-up views of **F** at the upper and lower parts of the kernel, respectively. The sections were stained with I_2KI and fast green. *p* pericarp; *n* nucellar membrane; *g* gap. Bars = 0.27 mm in **A**; 0.16 mm in **C**; 80 μm in **D**. The scale in **B**, **E**, and **F** is the same as in **A**. The scales in **G** and **H** are the same as in **C** and **D**, respectively

in the levels of soluble invertase activity in the glucose- and sucrose-based kernels which show, respectively, about 62 and 78% of the *in planta* levels of cell-wall invertase activity (Table 1). The fructose-based *Mn1* kernels, however, have shown coordinated control between the two invertase isozymes. Extremely low levels of enzyme activity in the *mn1* mutant (Table 2) do not allow a judicious evaluation of the mode of regulation in this genotype.

Immunohistological analysis of cell wall-bound invertase (CWI) in kernels cultured in vitro

Longitudinal sections of in vitro cultured kernels were utilized for localization of CWI protein at the cellular level. CWI in homozygous *Mn1* kernels, *in planta*, was restricted to endosperm transfer cells, one to two cell layers in the basal portion of the endosperm, as well as along the upper parts of the vascular bundles of the pedicel (Fig. 4 A and B; also see Cheng et al. 1996). Similar results were obtained when homozygous *Mn1* kernels were cultured for 14 days on sucrose, fructose, or glucose. Figures 4 C–F are longitudinal sections of *Mn1* kernels cultured on sucrose (Fig. 4 C and D) or fructose (Fig. 4 F). Cell wall-bound invertase was primarily present in a single cell layer of basal endosperm transfer cells (Fig. 4 C, D, and F), rather than the two cell layers as seen *in planta*. This presumably reflected the reduction, by about 20–30%, of enzyme activity in kernels cultured in vitro (Table 1). Relatively faint signals were seen in the upper parts of the vascular

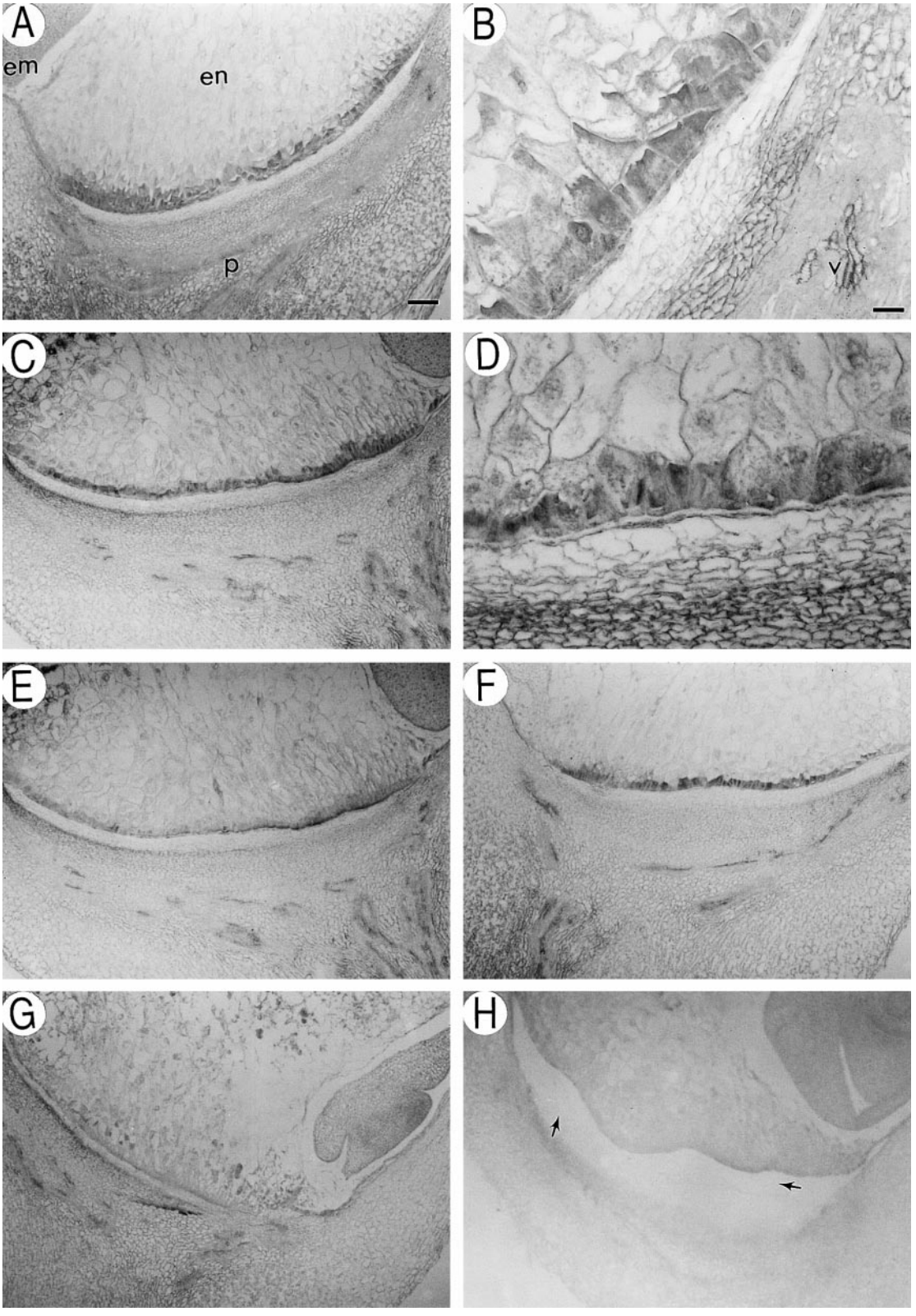

Fig. 4A–H Immunolocalization of cell wall-bound invertase (CWI) in kernels cultured on various sugar-supplemented media for 14 days. **A**, **B** CWI antibody treated section of homozygous *Mn1* kernels *in planta*. **B** is a close-up view of the section in **A**. **C**, **D** CWI antibody treated section of homozygous *Mn1* kernels grown on sucrose-supplemented medium. **D** is a close-up view of the section in **C**. **E** Pre-immune-treated longitudinal section of a homozygous *Mn1* kernel cultured on sucrose-supplemented medium. This section is from the same kernel as in **C**. **F** CWI antibody treated section of a homozygous *Mn1* kernel cultured on fructose-supplemented medium. **G** CWI antibody treated section of a homozygous *Mn1* kernel cultured on sugar-deprived medium. **H** CWI antibody treated section of a homozygous *mn1* kernel cultured on fructose-supplemented medium. *em* embryo; *en* endosperm; *p* pedicel; *v* vascular bundle; *arrow* points to the gap. Bars = 0.1 mm in **A**; 10 μm in **B**. The scale in **C**, **E**–**H**, and **D** are the same as in **A** and **B**, respectively

bundles of the pedicel (Fig. 4 F). No signal was detected in the pre-immune-treated control sections (Fig. 4 E), sections of *Mn1* kernels grown on sugar-deprived medium (Fig. 4 G), or invertase-deficient *mn1* kernels cultured on fructose (Fig. 4 H), sucrose or glucose (data not shown).

SDS Western-blot analyses of cell-wall invertase, sucrose synthases, and ADPGase proteins in kernels grown *in planta* and in vitro

Figure 5 illustrates the developmental profiles of the two isozymes of sucrose synthase, SS1 and SS2, and the small subunit of ADPGase which are known to play an important role in starch biosynthesis, in *Mn1* and *mn1* kernels, *in planta*. A marked reduction was seen in the levels of both SS isozymes in *mn1* kernels for all developmental stages during 12–24 DAP. Similarly, ADPGase levels were also reduced in the *mn1* mutant at 12 and 16 DAP, but slightly higher levels were seen at 24 DAP in the mutant than in the *Mn1* kernels (Fig. 5 A). Interestingly, an endosperm with one copy of the dominant *Mn1* allele [(i.e., *Mn1mn1mn1* obtained by the cross of *mn1mn1* (female) with *Mn1Mn1* (male)], reveals a wild-type phenotype with only about 20% of the total invertase activity of its male parent, and was sufficient to maintain near normal levels of the two sucrose synthase isozymes (SS1 and SS2) and the ADPGase proteins (Fig. 5 B) at 12 DAP. In fact, no reduction of sucrose synthases and ADPGase proteins was seen in an EMS-induced *mn1* mutant, *mn1-89*, which possess only aobut 6% total invertase activity and a 20% loss of seed weight. This indicates that invertase plays an important feed-forward regulatory role in down-regulating sucrose synthases and ADPGase expression at early developmental stages.

The same proteins were also examined in the two genotypes from kernels cultured in vitro for 14 days (Fig. 6). As expected from the enzyme activity data (Tables 1 and 2), no INCW2 protein was detected in the *mn1* mutant in both *in planta* and in vitro kernels. As for the SS isozymes and the ADPGase protein, in general the two genotypes were similar to each other and to the *in planta* kernels. However, there were certain subtle differences. In particular, the two SS isozymes were slightly reduced in the *Mn1* kernels cultured on hexose medium as compared to the *in planta* kernels or those cultured on sucrose. In the *mn1* genotype, the highest levels of both the SS isozymes were seen in kernels from sucrose-based medium relative to either the *in planta* or the hexose-based kernels. Levels of the ADPGase small subunit were slightly reduced in the *mn1*, compared to the *Mn1*, kernels for all the treatments. Similar expression patterns were seen for large subunit of ADPGase encoded by the *Sh2* gene in both *Mn1* and *mn1* kernels grown in vitro (data not shown).

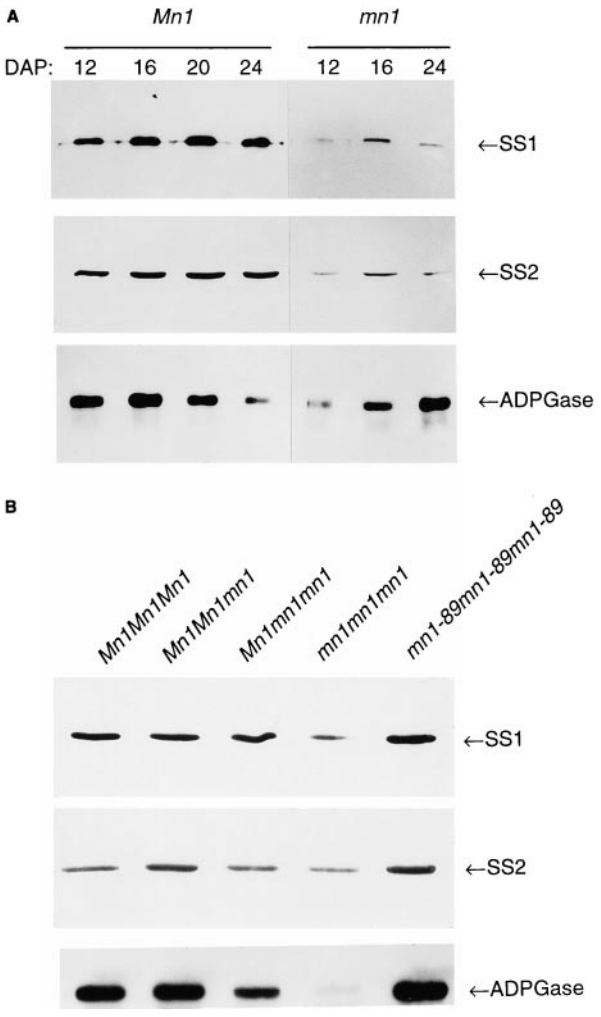

Fig. 5A, B SDS-protein gel blots showing sucrose synthases and ADPGase proteins in kernels at developmental stages and with various genotypes *in planta*. **A** Sucrose synthases and ADPGase proteins in kernels at various developmental stages. Each lane contains 5, 10, or 5 μg total protein of crude extracts for staining SS1, SS2, or the ADPGase small-subunit antiserum, respectively. **B** Sucrose synthases and ADPGase proteins in kernels with various genotypes at 12 DAP. Kernels with *Mn1Mn1mn1* were obtained by *Mn1Mn1* (female) × *mn1mn1* (male), whereas kernels with *Mn1 mn1mn1* were obtained by *mn1mn1* × *Mn1Mn1*. Each lane contained 5, 10, or 5 μg total protein of crude extracts for staining with SS1, SS2, or ADPGase antiserum, respectively

Discussion

Cell-wall invertase is essential for cell division in a developing endosperm

We have utilized a combined approach based on the in vitro kernel culture method (Gengenbach 1977) and a genetic tool, the invertase-deficient *mn1* seed mutant (Cheng et al. 1996), to elucidate certain early metabolic events in the utilization of sucrose in a developing seed in maize. As discussed previously, it is now well-estab-

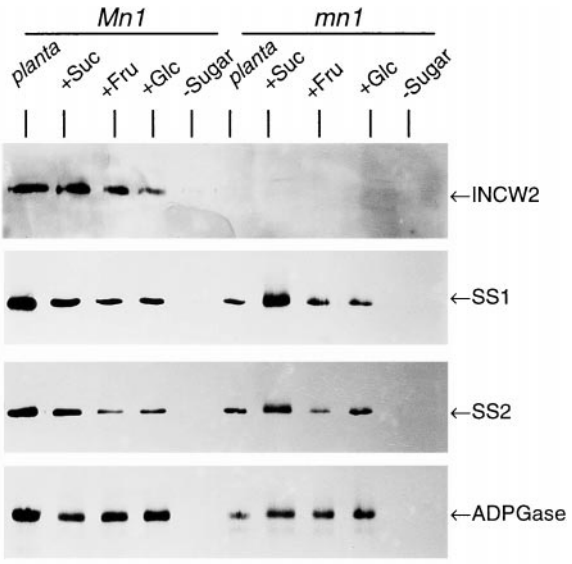

Fig. 6 SDS-protein gel blots showing various enzymes in crude extracts of wild-type and *mn1* kernel grown *in planta* and in vitro. Levels of proteins in μg are: INCW2 (50), SS1 (5), SS2 (10), and ADPGase (5)

lished that a substantial proportion (about 70%) of photo-assimilated sucrose enters a developing maize seed (Schmalstig and Hitz 1987; Felker 1992). Furthermore, our genetic studies on the *mn1* seed mutant indicate that the *Mn*1-encoded cell-wall invertase, the INCW2 enzyme, performs a critical role in the determination of sink strength, and ultimately the normal development of the seed (Cheng et al. 1996).

Because INCW2 is spatially and temporally the first enzyme to metabolize (i.e., hydrolyze) the sucrose entering an endosperm, it is reasonable to assume that hexoses (glucose and fructose) must be the physiological sugars for sugar sensing, the expression of the downstream metabolic genes and normal seed development, as is the case with many diverse organisms (Jang and Sheen 1997). The equivalent utilization of sucrose or a hexose sugar (glucose or fructose) in supporting normal growth under the in vitro kernel culture environment is consistent with such observations. Thus, it was expected that in vitro kernel culture of the *mn1* seed mutant in glucose, fructose or glucose + fructose medium would bypass the bottleneck caused by the invertase-deficiency of the mutant, and lead to a wild-type seed phenotype (i.e., a phenocopy). Surprisingly, no such phenocopies were recovered; the *mn1* seed mutant remained mutant regardless of the source of sugar, whereas the same sugars led to normal seed development of the *Mn1* kernels. The lack of a wild-type seed phenotype in the *mn1* mutant was confirmed by several criteria, including anatomical, biochemical and immunohistological data, all of which show no change relative to the *in planta mn1* kernels.

It is of particular interest to note from the kernel sections that the greatly reduced size of the *mn1* seed mutant was primarily due to a substantial reduction in its endosperm. Zhou et al. (1997) have observed half as many cells in *mn1* endosperm as in *Mn1*. We have shown previously that the highest level of invertase activity is at approximately 12 DAP (Cheng et al. 1996), a stage which coincides with the cell-division phase in maize endosperm (Kiesselbach 1949). Remarkably, Weber et al. (1996 a) have shown that greater CWI activity and high hexose levels in cotyledonary cells of *V. faba* are both correlated with extended mitotic activity. In addition, they have also shown that the ratio of hexose to sucrose is critical in determining whether the cells will continue dividing or begin differentiating. Similarly, an increased tuber size in transgenic potato is associated with an increase in the apoplastic expression of yeast invertase (Sonnewald et al. 1997). Collectively, these data from diverse plant sources show that the CWI plays a critical role in cell division in various sink tissues.

Our Western-blot data on *in planta* extracts indicate reduced levels of the SS proteins (Fig. 5) in the *mn1* as compared to the *Mn1* kernels. It is possible that an overall reduced cell surface area in the mutant endosperm leads to a feed-forward down-regulation of the *Sh1* and *Sus1* genes, respectively encoding the SS1 and SS2 proteins that play a critical role in both starch and cellulose biosynthesis in developing endosperm (Chourey et al. 1991 a; Carlson and Chourey 1996). Such cross-regulation leading to a down-regulation of the SS genes is most likely a secondary effect caused by the invertase-deficiency of the *mn1* seed mutant. This is clearly evidenced by the normal levels of SS proteins in the lineage-related *mn1-89* mutant and the *Mn1mn1* hybrid (where the *Mn1* parent is a pollen parent which contributes a single copy of the dominant gene to a triploid endosperm, *mn1mn1Mn1*), which have about 6 and 20% of wild-type invertase activity, respectively.

With respect to the starch levels, there was no major reduction in the mutant relative to the *Mn1* endosperm (Fig. 3); nor was there a reduction in the level of a key enzyme of starch biosynthesis, ADPGase protein, especially *in planta* at the 16 and 24 DAP stages, (Fig. 5). Thus, there appears to be a dichotomy in response to the sugar-based signals in maize endosperm, those which affect cell division and elongation were independent from those which control the storage function of starch biosynthesis.

Invertase-released hexoses play a functional role in normal seed development

An important observation of this study is the lack of restoration of a normal seed phenotype to the *mn1* seed mutant by in vitro kernel culture on hexose media. It is

significant that the same media led to a normal seed phenotype of the *Mn1* genotype. Why the *mn1* mutant seeds did not yield phenocopies of the wild-type seeds on hexose sugars is not clear. It is possible that hexoses might be re-synthesized to sucrose at some stage in the cellular pathway such that the invertase-deficiency remained a limiting step for the *mn1* mutant, but not the *Mn1* kernels. There is ample physiological evidence in maize that sucrose is cleaved and re-synthesized during its assimilation in a developing kernel (Shannon 1972; Porter et al. 1985; Hanft and Jones 1986; Felker 1992). However, the exact cellular site for such interconversion reactions has remained controversial. Our recent data show high levels of a sucrose-synthesizing enzyme, sucrose-phosphate synthase (SPS), entirely localized to the basal region in the developing endosperms of both *Mn1* and *mn1* kernels (Chourey et al. 1993; Cheng 1997). In addition, the basal endosperm also shows the SS2 protein that is associated with the plasma membrane (Chen and Chourey 1989; Carlson and Chourey 1996). Based on collective evidence, we suggest that such cleavage and re-synthesis of sucrose might occur in the basal region in a developing endosperm. If so, it is plausible that a metabolic release of hexose sugars in the basal endosperm apoplast, through sucrose hydrolysis, is a critical function from both a bioenergetic standpoint (i.e., a kinetically favorable downhill reaction) as well as in sensing and transducing signals for the expression of downstream genes engaged in carbon assimilation. Such a cycle of sucrose cleavage and re-synthesis, also known as a "futile cycle", has been well analyzed in suspension-cultured cells of *Chenopodium rubrum* (Dancer et al. 1990) and sugarcane (Wendler et al. 1990), as well as in germinating and developing cotyledons of *Ricinus communis* (Geigenberger and Stitt 1991) and *V. faba* (Weber et al. 1996 b). In fact, Stitt et al. (1995) have suggested that a "futile" cycle may regulate the net balance of carbohydrate flux into and out of the cell and may act as a controlling mechanism on the levels of carbon entering a sink tissue.

Remarkably, unlike the situation *in planta*, in vitro kernels have shown little or no difference between the *Mn1* and *mn1* genotypes in the expression of downstream genes encoding the SS1, SS2 and ADPGase proteins. Also lacking was the coordinate regulation between CWI and the soluble invertase activities from in vitro *Mn1* kernels obtained from sucrose or glucose-based medium. Such differences between *in planta* and in vitro culture environments are similar to the observations reported by Krapp et al. (1993) who have shown that carbohydrate content *per se* is not the direct signal for the regulation of gene expression in photosynthetic tissues; instead, an in vivo metabolism of sugars determines the appropriate regulation of downstream genes. Based on these data we suggest that the apoplastic cleavage of sucrose, mediated by the *Mn1*-encoded INCW2, is essential for normal regulation of the downstream genes.

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References

Carlson SJ, Chourey PS (1996) Evidence for plasma membrane-associated forms of sucrose synthase in maize. Mol Gen Genet 252: 303–310

Chen Y-C, Chourey PS (1989) Spatial and temporal expression of the two sucrose synthase genes in maize: Immunohistochemical evidence. Theor Appl Genet 78: 553–559

Cheng W-H (1997) Gene-expression analysis of acid invertase and sucrose-phosphate synthase in maize. Ph D dissertation, University of Florida, pp 109–134

Cheng W-H, Taliercio EW, Chourey PS (1996) The *Miniature1* seed locus of maize encodes a cell-wall invertase required for normal development of endosperm and maternal cells in the pedicel. Plant Cell 8: 971–983

Chourey PS, Chen, Y-C, Miller ME (1991 a) Early cell degeneration in developing endosperm is unique to the *shrunken* mutation in maize. Maydica 36: 141–146

Chourey PS, Taliercio EW, Kane EJ (1991 b) Tissue-specific expression and anaerobically induced post-transcriptional modulation of sucrose synthase genes in *Sorghum bicolor* M. Plant Physiol 96: 485–490

Chourey PS, Taliercio EW, Im KH (1993) Sucrose phosphate synthase in developing kernel of maize (abstract). Plant Physiol 102 (suppl): 24

Cobb BG, Hannah LC (1986) Sugar utilization by developing wild-type and *shrunken-2* maize kernels. Plant Physiol 80: 609–611

Cobb BG, Hannah LC (1988) *Shrunken-1*-encoded sucrose synthase is not required for sucrose synthesis in the maize endosperm. Plant Physiol 88: 1219–1221

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

Dancer J, Hatzfeld WD, Stitt M (1990) Cytosolic cycles regulate the turnover of sucrose in heterotrophic cell-suspension cultures of *Chenopodium rubrum* L. Planta 182: 223–231

Doehlert DC, Felker FC (1987) Characterization and distribution of invertase activity in developing maize (*Zea mays*) kernels. Physiol Plant 70: 51–57

Felker FC (1992) Participation of cob tissue in the uptake of medium components by maize kernels cultured in vitro. J Plant Physiol 139: 647–652

Geigenberger P, Stitt M (1991) A futile cycle of sucrose synthesis and degradation is involved in regulating partitioning between sucrose, starch and respiration in cotyledons of germinating *Ricinus communis* L. seedlings when phloem transport is inhibited. Planta 85: 812–90

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

Gengenbach BG, Green CE (1975) Selection of T-cytoplasm maize callus cultures resistant to *Helminthosporium maydis* race T pathotoxin. Crop Sci 15: 645–649

Giroux MJ, Hannah LC (1994) ADP-glucose pyrophosphorylase in *shrunken-2* and *brittle-2* mutants of maize. Mol Gen Genet 342: 400–408

Griffith SM, Jones RJ, Brenner ML (1987) In vitro sugar transport in *Zea mays* L. kernels. Plant Physiol 84: 467–471

Hanft JM, Jones RJ (1986) Kernel abortion in maize. II. Distribution of ^{14}C among kernel carbohydrates. Plant Physiol 81 : 511–515

Jang J-C, Sheen J (1997) Sugar sensing in higher plants. Trends Plant Sci 2 : 208–214

Kiesselbach TA (1949) The structure and reproduction of corn. In: Research Bulletin 161, Agricultural Experimental Station. University of Nebraska Press, Lincoln, NE

Krapp A, Hoffman B, Schafer C, Stitt, M (1993) Regulation of the expression of *rbc*S and other photosynthetic genes by carbohydrates. A mechanism for the 'sink regulation' of photosynthesis? Plant J 3 : 817–828

Lowe J, Nelson OE (1946) Miniature seed – a study in the development of a defective caryopsis in maize. Genetics 31 : 525–533

Miller ME, Chourey PS (1992) The maize invertase-deficient *miniature-1* seed mutation is associated with aberrant pedicel and endosperm development. Plant Cell 4 : 297–305

Nelson N (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem 153 : 375–380

Porter GA, Knievel DP, Shannon JC (1985) Sugar efflux from maize (*Zea mays* L.) pedicel tissue. Plant Physiol 77 : 524–521

Schmalstig JG, Hitz WD (1987) Transport and metabolism of a sucrose analog (1′-fluorosucrose) into *Zea mays* L. endosperm without invertase hydrolysis. Plant Physiol 85 : 902–905

Shannon JC (1972) Movement of [^{14}C]-labeled assimilates into kernels of *Zea mays* L. I. Pattern and rate of sugar movement. Plant Physiol 49 : 198–202

Shannon JC, Knievel DP, Chourey PS, Liu S-Y, Liu KC (1993) Carbohydrate metabolism in the pedicel and endosperm of miniature maize kernels (abstract). Plant Physiol 102 (suppl): 42

Shimamoto K, Nelson OE (1981) Movement of ^{14}C compounds from maternal tissue into maize seeds grown in vitro. Plant Physiol 67 : 429–432

Smeekens S, Rook F (1997) Sugar sensing and sugar-mediated signal transduction in plants. Plant Physiol 115 : 7–13

Sonnewald U, Hajirezaei M-R, Kossmann J, Heyer A, Trethewey RN, Willmitzer L (1997) Increased potato tuber size resulting from apoplastic expression of a yeast invertase. Nature Biotechnol 15 : 794–797

Stitt M, Krapp A, Klein D, Röper-Schwarz A, Paul M (1995) Do carbohydrates regulate photosynthesis and allocation by altering gene expression? In: Madore MA, Lucas WJ (eds) Carbon partitioning and source-sink interaction in plants. American Society of Plant Physiologists, Rockville, Maryland, pp 68–77

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

Weber H, Borisjuk L, Wobus U (1996 a) Controlling seed development and seed size in *Vicia faba*: a role for seed coat-associated invertases and carbohydrate state. Plant J 10 : 823–834

Weber H, Buchner P, Borisjuk L, Wobus U (1996 b) Sucrose metabolism during cotyledon development of *Vicia faba* L. is controlled by the concerted action of both sucrose phosphate synthase and sucrose synthase: expression patterns, metabolic regulation and implications for seed development. Plant J 9 : 841–850

Weber H, Borisjuk L, Heim U, Sauer N, Wobus U (1997 a) A role for sugar transporters during seed development: molecular characterization of a hexose and a sucrose carrier in fava bean seeds. Plant Cell 9 : 895–908

Weber H, Borisjuk L, Wobus U (1997 b) Sugar import and metabolism during seed development. Trends Plant Sci 2 : 169–174

Wendler R, Veith R, Dancer J, Stitt M, Komor E (1990) Sucrose storage in cell suspension cultures of *Saccharum* is regulated by a cycle of synthesis and degradation. Planta 183 : 31–39

Zhou Y, Knievel DP, Shannon JC (1997) Disruption of kernel growth and development in the miniature maize mutant (abstract) Plant Physiol 114 (Suppl): 78