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The *Miniature1* Seed Locus of Maize Encodes a Cell Wall Invertase Required for Normal Development of Endosperm and Maternal Cells in the Pedicel

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Collective evidence demonstrates that the *Miniature1* (*Mn1*) seed locus in maize encodes an endosperm-specific isozyme of cell wall invertase, CWI-2. The evidence includes (1) isolation and characterization of ethyl methanesulfonate-induced *mn1* mutants with altered enzyme activity and (2) a near-linear relationship between gene/dose and invertase activity and the CWI-2 protein. In addition, molecular analyses showed that the cDNA clone *Incw2* maps to the *Mn1* locus and differentiates the six ethyl methanesulfonate-induced *mn1* mutants of independent origin into two classes when RNA gel blot analyses were used. We also report two unexpected observations that provide significant new insight into the physiological role of invertase and its regulation in a developing seed. First, a large proportion of total enzyme activity (~90%) was dispensable (i.e., nonlimiting). However, below the threshold level of ~6% of wild-type activity, the endosperm enzyme controlled both the sink strength of the developing endosperm as well as the developmental stability of maternal cells in the pedicel in a rate-limiting manner. Our data also suggest an unusually tight coordinate control between the cell wall-bound and the soluble forms of invertase, which are most likely encoded by two separate genes, presumably through metabolic controls mediated by the sugars.

INTRODUCTION

The enzyme invertase (EC 3.2.1.26), also known as β-fructofuranosidase, is known to catalyze sucrose breakdown to glucose and fructose in an irreversible manner. It is one of the oldest known enzymes in classical enzymology and is well studied in terms of its biochemical and physiological properties in numerous plants. At least two forms of the enzyme, soluble and particulate, are a common feature to all of the invertases. The soluble form is predominantly localized in vacuoles and cytoplasm. The particulate form is ionically bound to the cell wall (hence, cell wall–bound form) and is readily extractable with a high salt concentration (Sturm and Chrispeels, 1990; von Schaewen et al., 1990; Weil et al., 1994). Each of the two forms of invertase is known to have several isozymes (Jaynes and Nelson, 1971; Unger et al., 1994).

Recently, a number of invertase genes encoding cell wall–bound and soluble forms of the enzyme have been cloned from a diverse group of plant species (Sturm and Chrispeels, 1990; Elliot et al., 1993; Ramloch-Lorenz et al., 1993; Greiner et al., 1995; Roitsch et al., 1995; Weber et al., 1995). Two important features have emerged from these studies. First, there are several genes that encode various isozymes of invertases (i.e., a small gene family); in fact, it is probable that each tissue in a plant may have a unique set of genes for the two isozymes. Second, the predicted amino acid sequences for cell wall–bound and soluble forms of the enzyme suggest that the two proteins belong to two distinct classes. Much more sequence similarity is seen among the members of each class from diverse taxonomic groups than is seen between each class represented from the same plant group (Unger et al., 1994; Weber et al., 1995). Similarly, in maize, the deduced amino acid sequences of the cDNA clone of a cell wall invertase, CWI-1, isolated from a cell suspension culture library (Shanker et al., 1995), shares 59.1% sequence identity with the carrot CWI cDNA (Sturm and Chrispeels, 1990), whereas it shares only 44.7% identity with the maize soluble invertase clone reported by Xu et al. (1995). A similar sequence comparison of the maize CWI-2 protein, predicted from a cDNA clone isolated from developing endosperm library, has 70.5% sequence identity with CWI-1 (Taliercio et al., 1995; E.W. Taliercio, S. Shanker, J.-H. Choi, and P.S. Chourey, manuscript in preparation).

The physiological role of invertases is believed to be in sucrose partitioning between source and sink regions of the plant (Eschrich, 1980). Although the experimental basis for such a role is largely speculative and correlative in nature, the most significant data are now emerging from studies with transgenic

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tomato (Dickinson et al., 1991) and tobacco (von Schaewen et al., 1990) plants overexpressing CWI in a constitutive fashion. Elevated levels of enzyme activity in such plants cause reduced levels of sucrose transport between sink and source tissues and lead to stunted growth and overall altered plant morphology. In this regard, the *miniature1* (*mn1*) seed mutant in maize, first described by Lowe and Nelson (1946), is of special interest for several reasons. Most importantly, it is the first invertase-deficient mutant identified in plants (Miller and Chourey, 1992). The biochemical lesion is endosperm specific, as is the seed phenotype, and as the name implies, the mutant is marked by a drastic reduction in endosperm weight and size relative to that of the wild type, *Mn1*. Another feature of the *mn1* seed mutant, also first reported by Lowe and Nelson (1946), is an early withdrawal of the pedicel from the developing endosperm at ~9 to 10 days after pollination (DAP). Consequently, the developing endosperm is starved for nutrients from the mother plant, and its subsequent growth and development are reduced drastically. Invertase activity in a normal developing kernel is localized entirely in the basal third of the endosperm and pedicel (Doehlert and Felker, 1987). Significantly, the *mn1* seed is deficient in invertase activity in both filial endosperm and the maternal pedicel tissue. Our genetic analyses suggest that the loss of enzyme activity in endosperm is the causal basis of its loss in the pedicel because there is an actual physical destruction of cells, leading to a gap formation between pedicel and endosperm. The *mn1* seed mutant is thus unique because a single gene mutation affects both the filial as well as the maternal generations of the plant.

In this study, we present several lines of collective evidence showing that the *Mn1* locus encodes an endosperm-specific invertase—in particular, a cell wall invertase designated CWI-2. In addition, we show an unusually tight coordinate control in the levels of cell wall and soluble forms of invertase activities, presumably due to metabolic regulation by a common substrate, sucrose. Finally, the data also indicate that a substantial proportion of invertase activity, ~90% of wild-type levels, was dispensible without a detectable change in seed phenotype. However, reductions below the threshold levels led to both the loss of sink strength of developing endosperm as well as the developmental stability of placento-chalazal cells in the pedicel in a rate-limiting manner.

RESULTS

Invertase Activity during Endosperm Development

Figure 1A presents developmental profiles of both cell wall and soluble forms of invertases in terms of specific activity. The highest level of specific activity for both forms of the enzyme was at 12 DAP. Although there was a gradual decline of invertase activities in subsequent developmental stages, a residual level of ~25 to 30% of the highest level was still detectable

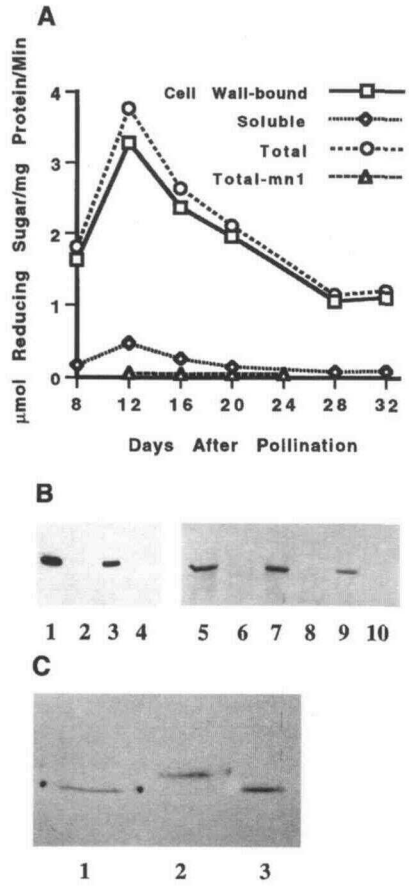

Figure 1. Developmental Profile of Invertase Activity and Protein in Immature Kernels.

(A) Specific activity values are shown for crude extracts of homozygous *Mn1* kernels harvested at various developmental stages. The highest level of activity was at 12 DAP for *Mn1* kernels. *mn1-1* kernels had only low to undetectable levels of activity at 12 to 24 DAP. Values represent means of duplicate measurements with standard deviations <4%; the results are reproducible in at least two sets of independent experiments.

(B) Protein gel blot analysis on an SDS–polyacrylamide gel shows the p72 CWI polypeptide in kernel extracts from lower (odd numbers) and upper (even numbers) parts of kernels harvested at 12 (lanes 1 and 2), 16 (lanes 3 and 4), 24 (lanes 5 and 6), 28 (lanes 7 and 8), and 32 (lanes 9 and 10) DAP. Each lane represents 15 (lanes 1 and 3) and 60 μg (lanes 2, 4, and 5 to 10) of protein in crude extracts, respectively.

(C) Protein gel blot analysis on an SDS–polyacrylamide gel shows the CWI polypeptide in extracts from the pedicel (lane 1), whole kernel (12 DAP) without the pedicel (lane 2), and suspension culture cells (lane 3). The amount of protein in crude extracts is as follows: lane 1, 80 μg; lane 2, 40 μg; lane 3, 50 μg. The CWI polypeptide in the pedicel and suspension culture cell extracts is estimated as ~68 kD.

during the advanced stages at 28 to 32 DAP. Of the two forms of invertase activities, the cell wall form was predominant, contributing almost 90% of the total invertase activity. In the *mn1-1* (the reference allele) seed mutant, both forms of enzyme activities remained low to undetectable during the entire period of endosperm development.

Figure 1B shows protein gel blots on SDS–polyacrylamide gels of CWI polypeptides in crude extracts from the upper two-thirds and lower one-third of a developing kernel at various developmental stages. The CWI polypeptides were detected using polyclonal antibodies raised against carrot CWI, which is specific for only the CWI protein and does not cross-react with the soluble invertases (Unger et al., 1992). A polypeptide of 72 kD (p72) was seen in extracts from only the lower parts of the kernel throughout the entire period of kernel development. Figure 1C presents a protein gel blot similar to the one shown in Figure 1B. It illustrates that a CWI polypeptide in extracts from the pedicels of 12-DAP kernels and maize suspension cells is slightly smaller, ~68 kD (p68), than that in the endosperm.

Gene-Dose Relationship with Invertase Activity

To understand better the genetic basis of the loss of invertase activity in the *mn1* seed mutant (Miller and Chourey, 1992), we have now isolated and analyzed six new *mn1* seed mutants generated by ethyl methanesulfonate (EMS) mutagenesis (see Methods for details). Of these six mutants, one mutant, *mn1-89*, is of special interest and is the focus of detailed analyses reported below. Table 1 presents comparative levels of both cell wall–bound and soluble forms of invertase activities in kernel extracts from homozygous *Mn1*, *mn1-89*, and *mn1-1* and the heterozygous genotypes obtained by reciprocal crosses among *Mn1* and *mn1- 1*, *Mn1* and *mn1-89*, and *mn1-89* and *mn1-1* parents. Three major observations are noteworthy.

First, the homozygous *mn1-89* mutant, as compared with the *mn1-1* mutant, showed a significant increase in both cell wall–bound and soluble forms of invertase activities. Specifically, it had an approximately fourfold increase in invertase activity relative to the *mn1-1* mutant. However, as compared with the wild type, both *mn1-89* and *mn1-1* mutants had only ~6 and 1.5% of the levels of wild-type activity, respectively. Second, there is a linear relationship between the level of total invertase activity and the number of *mn1-89* alleles in triploid endosperm genotypes representing three, two, and one copies of the *mn1-89* alleles, with three copies having the most and one the least amount. A similar relationship was also seen in reciprocal hybrids between *Mn1* and *mn1-89* and *Mn1* and *mn1-1* parents, with the exception that the hybrid with a single copy of the *Mn1* allele, *Mn1/mn1-89/mn1-89*, had lower than the expected levels of ~35% based on a linear relationship. In each set of hybrids, CWI activities were closer to the expected linearity than the soluble form of the enzyme activity. And finally, the cell wall–bound and soluble forms of invertase activities appear to be coordinately controlled in various genotypes. Specifically, in each case, a relative increase or decrease in levels of the predominant cell wall form was always associated with a correspondingly similar change in levels of the soluble form.

Figure 2 shows protein gel blots on SDS–polyacrylamide gels with a CWI-specific polypeptide detectable in crude extracts from either the whole kernel (Figure 2A) or the lower third of the kernel (Figures 2B and 2C) in two sets of parents—(1) *Mn1–mn1-1* (Figures 2A and 2B) and (2) *Mn1–mn1-89* (Figure 2C)—and the reciprocal hybrids between each set of parents. The p72 polypeptide was readily detectable in all genotypes, except for the *mn1-1* mutant (Figures 2A and 2B) and the five newly induced mutant alleles (data not shown). There was also an increase in the intensity of this band in extracts from the lower third of the kernel when compared with the whole kernel in all genotypes (Figures 2B and 2A, respectively). This is in agreement with previous data showing a preferential localization of enzyme activity to only the lower part of the endosperm (Doehlert and Felker, 1987; Miller and Chourey, 1992). Similar extracts from upper parts of the kernel showed no detectable levels of p72 (data not shown). Noteworthy is that crude extracts from the *mn1-89* homozygote had a faint band of the p72 protein, which was undetectable in the *mn1-1*

Table 1. Gene–Dose Relationship with Enzyme Activity in Various Genotypes of Immature Kernels at 12 DAP

	Enzyme Activity[a] (μmol reducing sugar/mg protein/min)		
Genotype	Bound (%)[b]	Solu.[c] (%)	Total (%)
mn1-89 versus *mn1-1*			
Mn1Mn1Mn1	1.752 (100)	0.225 (100)	1.980 (100)
mn1-89mn1-89mn1-89	0.084 (4.8)	0.033 (14.7)	0.117 (5.9)
mn1-89mn1-89mn1-1	0.052 (3.0)	0.025 (11.4)	0.077 (3.9)
mn1-89mn1-1mn1-1	0.025 (1.4)	0.019 (8.4)	0.044 (2.2)
mn1-1mn1-1mn1-1	0.020 (1.1)	0.008 (3.6)	0.028 (1.4)
Mn1 versus *mn1-1*			
Mn1Mn1Mn1	1.516 (100)	0.276 (100)	1.792 (100)
Mn1Mn1mn1-1	1.232 (81.2)	0.176 (63.8)	1.408 (78.6)
Mn1mn1-1mn1-1	0.227 (15.0)	0.150 (54.3)	0.377 (21.0)
mn1-1mn1-1mn1-1	0.022 (1.4)	0.011 (4.0)	0.033 (1.8)
Mn1 versus *mn1-89*			
Mn1Mn1Mn1	1.819 (100)	0.191 (100)	2.010 (100)
Mn1Mn1mn1-89	1.149 (63.4)	0.171 (89.5)	1.320 (65.7)
Mn1mn1-89mn1-89	0.283 (15.6)	0.114 (59.7)	0.397 (19.8)
mn1-89mn1-89mn1-89	0.078 (4.3)	0.034 (17.8)	0.112 (5.6)

[a] Values represent means of duplicate measurements with standard deviation below 2%; the results are reproducible in at least two sets of independent experiments.

[b] Values within parentheses are normalized to homozygous *Mn1* genotype.

[c] Solu., soluble.

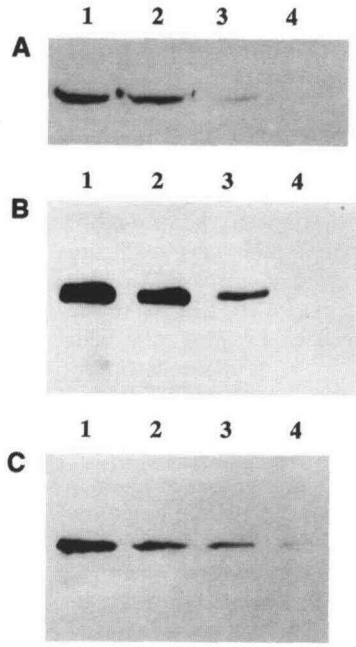

Figure 2. Protein Gel Blots Showing a Positive Correlation between Gene Dose at the *Mn1* Locus and the Levels of CWI Protein in 12-DAP Kernels.

(A) Each lane contains 50 μg of protein in crude extracts from whole kernels of *Mn1/Mn1/Mn1* (lane 1), *Mn1/Mn1/mn1-1* (lane 2), *Mn1/mn1-1/mn1-1* (lane 3), and *mn1-1/mn1-1/mn1-1* (lane 4) homozygotes or hybrids.
(B) The same gel as shown in **(A)**, except only the lower third of the kernels was used for preparing the extracts.
(C) Each lane contains 50 μg of protein in crude extracts from the lower third of kernels of *Mn1/Mn1/Mn1* (lane 1), *Mn1/Mn1/mn1-89* (lane 2), *Mn1/mn1-89/mn1-89* (lane 3), and *mn1-89/mn1-89/mn1-89* (lane 4) homozygotes or hybrids.
A faint band representing extremely low levels of CWI protein was detected in homozygous *mn1-89* but not in the *mn1-1* mutant.

extracts (Figures 2C and 2B, respectively). These differences between the two mutants are consistent with the enzyme activity data; the *mn1-89* mutant had approximately fourfold higher levels of cell wall–bound invertase activity than did the *mn1-1* mutant. We speculate that the residual low level of enzyme activity in the *mn1-1* extracts was below the detection limits of the immunoblot using the heterologous antibody. The results in Figure 2 also demonstrate that band intensities corresponding to p72 are correlated with the number of *Mn1* gene copies in the various genotypes. Although the band intensities were not quantified, these are in qualitative agreement with the levels of enzyme activities shown in Table 1.

Immunolocalization of Invertase in Kernel Sections

As shown in Figure 3, light microscopy was used to determine the immunolocalization of the CWI protein in longitudinal sections of 12- to 16-DAP kernels of various genotypes. Because there was no signal in the upper part of the kernels, only the lower parts of kernels, including the endosperm and pedicel, were included in these analyses. A positive signal, evidenced by an intense fuchsia-colored reaction product, was readily detectable in *Mn1*, but not in *mn1-1* kernels (Figures 3B and 3F, respectively), and in *Mn1* kernels treated with the preimmune serum (Figure 3A). There also was no signal in *mn1-89* kernel sections (Figure 3E); the low levels of p72 determined by protein gel blot analyses were presumably below the detection limits of this method. The positive signal seen in *Mn1* sections was limited to the apoplastic region and to wall ingrowths of the basal endosperm transfer cells (Figures 3C and 3D). We have also examined a nonallelic mutant, *miniature2* (*mn2*), that has a seed phenotype similar to the *mn1* seed mutant. A positive signal in the *mn2* sections (Figure 3G) is consistent with our data showing normal levels of invertase activity and the p72 protein by using protein blot analyses (data not shown). A low-level signal was also detectable in pedicels along vascular bundle regions of the *Mn1* kernels (Figures 3B and 3C). The staining intensity in tissue sections was consistent with the low levels of p68 protein in pedicel extracts shown in Figure 1C (lane 1).

Developmental Stability of Pedicel and Seed Size

The causal basis for the withdrawal of pedicel from developing endosperm is believed to be the lack of invertase activity in the *mn1* endosperm (Miller and Chourey, 1992). To understand this unusual interaction better, we have now examined kernel sections of the homozygous parents, *mn1-89* and *mn1-1*, and their reciprocal hybrids at 12 to 16 DAP, primarily because these genotypes have the lowest levels of endosperm invertase activities reported thus far (Table 1). The results from this anatomical study are shown in Figure 4. The homozygous *mn1-89* parent and the hybrid with two copies of the *mn1-89* alleles (*mn1-89/mn1-89/mn1-1*) did not develop a detectable gap between the endosperm and pedicel (Figures 4A and 4B, respectively). In contrast, the reciprocal heterozygote with a single copy of the *mn1-89* allele (*mn1-89/mn1-1/mn1-1*) was indistinguishable from the *mn1-1* homozygote; a large gap was readily detectable in these two genotypes (Figures 4C and 4D). Thus, the withdrawal of the pedicel from the developing endosperm, or its lack thereof, is clearly correlated with a certain threshold level of invertase activity in the endosperm.

Figure 5 presents ears of several genotypes to show comparative seed phenotypes at maturity; in particular, those representing the various doses of the *mn1-89* allele are shown. Homozygous *mn1-89* seed (Figure 5, second ear) are strikingly similar to those of the wild type (Figure 5, first ear); the *mn1-89* seed display neither the drastic reductions in seed size nor the appearance of loose and papery pericarp typifing the *mn1-1* seed mutant. Interestingly, the reciprocal hybrids representing two copies and one copy of the *mn1-89* allele in the endosperm genome but the same embryo genotype,

mn1-89/mn1-1, are also readily distinguishable from each other. Seed of the former hybrid (Figure 5, third ear) are intermediate in size relative to the two parents, *mn1-89* and *mn1-1*, whereas the latter (Figure 5, fourth ear) are indistinguishable from the *mn1-1* parent (data not shown). Thus, the *mn1-89* allele is semidominant to *mn1-1* because the seed phenotype in the hybrid *mn1-89/mn1-1* depends on whether the endosperm carries one or two copies of the *mn1-89* allele. In addition, these genotypes also show a difference in the intensity of anthocyanin pigment in aleurone layers of mature seeds. The highest and lowest intensities of anthocyanin pigment were seen in homozygous *Mn1* and *mn1-1*, respectively. Color intensities in the rest of the genotypes were dependent on the number of *mn1-89* alleles in their aleurone layer and can be best described as *mn1-89/mn1-89/mn1-89* > *mn1-89/mn1-89/mn1-1* > *mn1-89/mn1-1/mn1-1*. Perhaps, the carbon moiety of anthocyanin compounds in the triploid aleurone layer is also limited by CWI-2 activity, especially in the low range of 6 to 1.5% of wild-type enzyme activity.

To determine whether these distinct seed phenotypes are correlated with seed weights (i.e., sink strength), we studied F_2 generation ears that were segregating for the homozygous *mn1-1* or *mn1-89* kernels as well as for the wild-type *Mn1* kernels on the same ear (see Methods). Relative seed weights, expressed as percentage values of wild-type kernels, were 36 and 77% for homozygous *mn1-1* and *mn1-89* kernels, respectively. Appropriate testcrosses were also made (see Methods) to obtain kernels with one or two copies of the *mn1-89* allele (i.e., *mn1-89/mn1-1/mn1-1* and *mn1-89/mn1-89/mn1-1*, respectively) segregating with wild-type kernels on the same ear. Relative seed weights associated with one or two copies of the *mn1-89* allele were 43 and 71% that of the wild type, respectively. Overall, these seed weight values are in good agreement with the seed phenotypes.

The *incw2* cDNA Clone Discriminates among Various *mn1* Mutants, as Shown by RNA Gel Blot Analysis, and Maps to the *Mn1* Locus

Figure 6A illustrates a gel blot showing steady state levels of CWI RNAs from 12-DAP kernels of various genotypes representing *Mn1*, *mn1-1*, and several EMS-induced *mn1* mutants. The membrane was hybridized with a radiolabeled full-length cDNA clone of maize CWI, *incw2*, previously isolated from a cDNA library of RNAs from only the lower parts of 12-DAP endosperm. The library was screened using a maize *incw1* cDNA clone, which represents a nonallelic gene on chromosome 5 (Shanker et al., 1995) and shows a distinctive tissue-specific expression pattern relative to *incw2* (E.W. Taliercio, S. Shanker, J.-H. Choi, and P.S. Chourey, manuscript in preparation). A 2.2-kb transcript was seen in RNAs from *Mn1* (lane 7) and three of the six EMS-induced *mn1* mutants, *mn1-74*, *mn1-84*, and *mn1-89* (lanes 3, 5, and 6, respectively); however, no *incw2* transcripts were seen in RNA from *mn1-1* (lane 2) and the three EMS-induced mutants, *mn1-82* (data not shown), *mn1-83* (data not shown), and *mn1-88* (lane 4). The *incw2* transcripts also were not detectable in a maize cell suspension culture (lane 1), which otherwise showed abundant levels of *incw1* RNA when *incw1* cDNA was used as a probe (E.W. Taliercio, S. Shanker, J.-H. Choi, and P.S. Chourey, manuscript in preparation).

Genetic mapping of the *incw2* clone was done at the University of Missouri (Columbia) Restriction Fragment Length Polymorphism laboratory, using an "immortalized" F_2 population (Gardiner et al., 1993). The *incw2* clone mapped to chromosome 2, showing no recombination with another marker, *php10012*, placed very close to *mn1* (Coe et al., 1995; E.H. Coe, personal communication). By using genetic mapping, only an approximate position for *mn1* in this region could be determined. In addition, we also examined genomic DNAs of lineage-related (W22 inbred line) individual seedlings of homozygous *Mn1*, *mn1-1*, and *mn1-89* genotypes derived from the F_3 generation of our aforementioned EMS experiment. Figure 6B illustrates a representative genomic DNA gel blot of BamHI-digested DNAs from such a population and the parental *mn1-1* strain of unknown genetic background (lane 4), which was the male parent in the mutation experiment. Genomic gel blots were hybridized with a radiolabeled full-length *incw2* cDNA clone. An ~10.5-kb BamHI fragment associated with the *mn1-1* parental strain (Figure 6B, lanes 3 and 4) cosegregated in 40 individual *mn1-1* seedlings examined so far. In contrast, an ~7.4-kb fragment seen at the *Mn1* locus (lane 1) cosegregated in 30 individual seedlings of the *mn1-89* mutant (lane 2).

DISCUSSION

Collective Evidence That *Mn1* Encodes a CWI

Several lines of evidence are presented here to demonstrate that the *Mn1* locus encodes an endosperm-specific cell wall invertase, CWI-2. This includes genetic evidence provided by the isolation of six EMS-induced *mn1* mutants, five of which are phenotypically indistinguishable from the naturally occurring *mn1-1* seed mutant. These five mutants are also biochemically indistinguishable from the *mn1-1* mutant, as determined by the enzyme deficiency and the lack of CWI-2 polypeptide in immunoblot analyses. The sixth mutant, *mn1-89*, however, was unique. Unlike *mn1-1*, which showed a loss of ~70% seed weight, homozygous *mn1-89* kernels showed only ~20% loss of seed weight and leaky expression, as determined by biochemical analyses. Such concomitant changes at the biochemical level in mutants selected on the basis of *mn1* seed phenotype strongly support our hypothesis that the *Mn1* locus encodes the CWI enzyme. A similar situation was previously demonstrated at the *shrunken1* (*Sh1*) locus in maize where EMS-induced mutations selected on the basis of *sh1* seed phenotype led to simultaneous changes in the

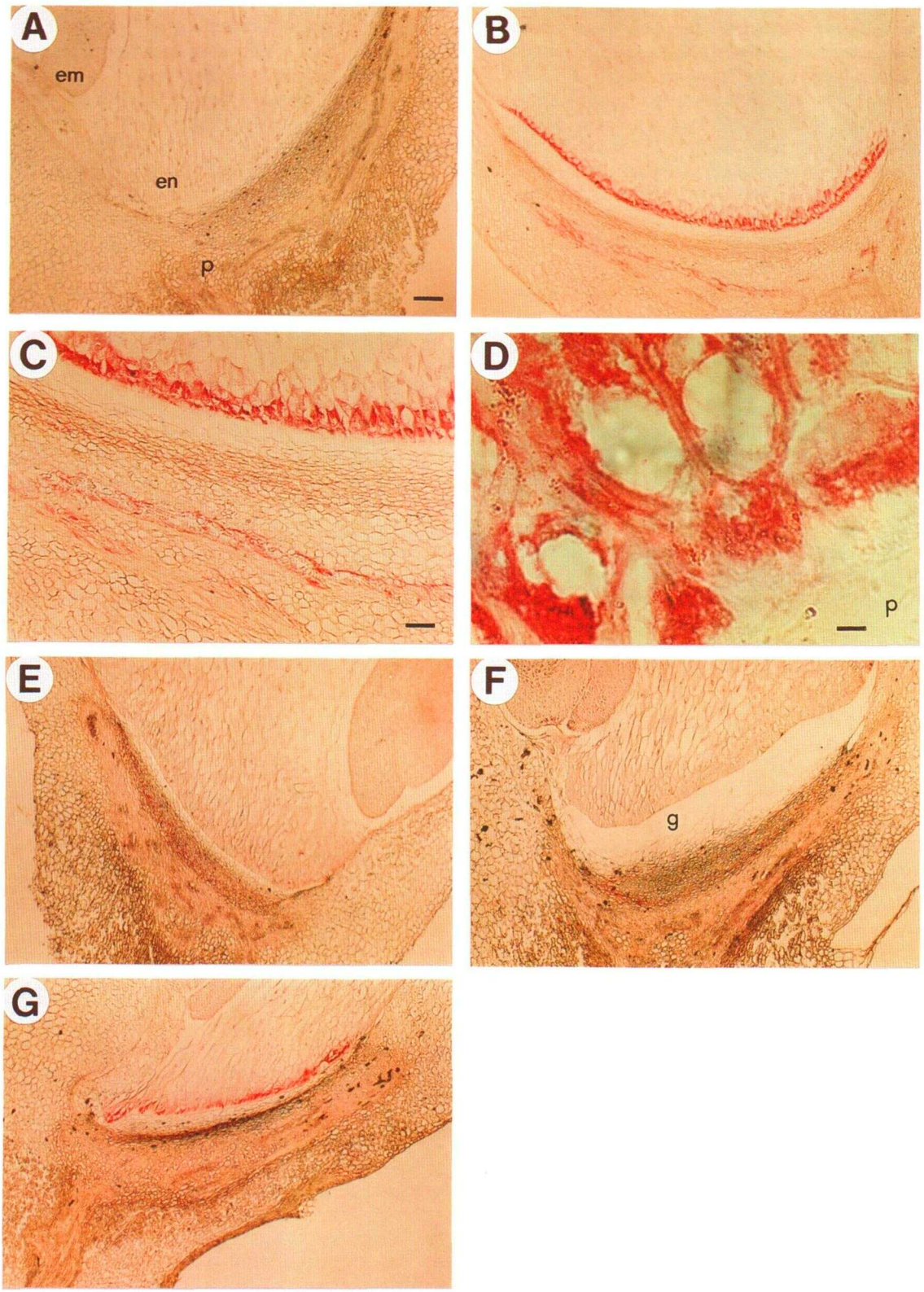

Figure 3. Immunolocalization of CWI Protein in 12-DAP Basal Endosperms and Pedicels in Various Genotypes.

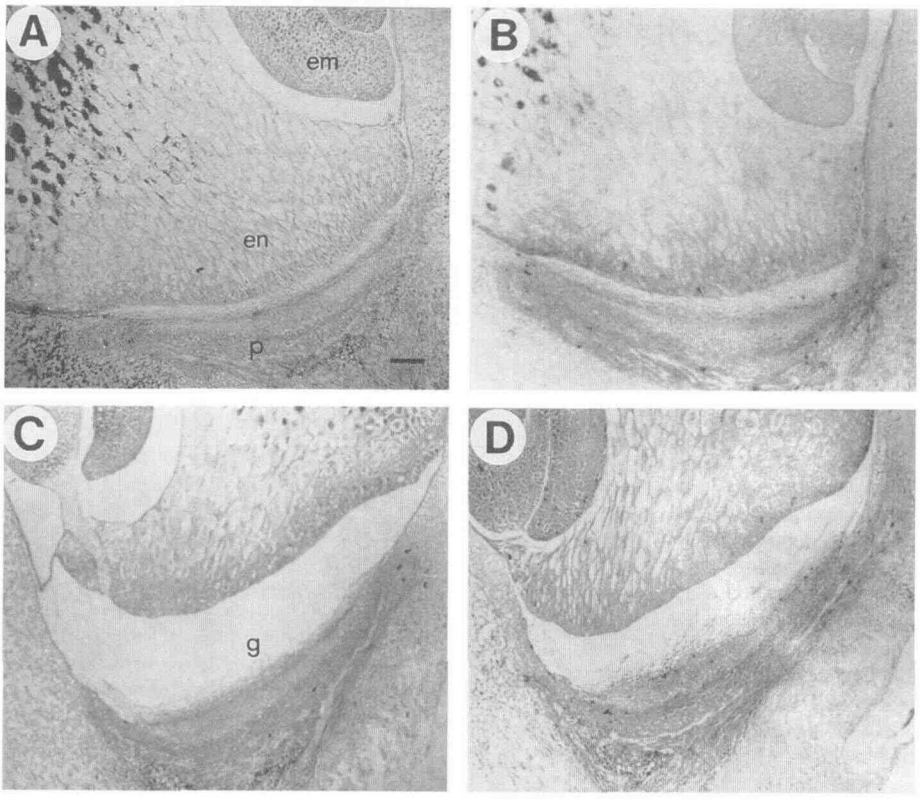

Figure 4. Relationship between the Number of the *mn1-89* Gene Copies and the Anatomical Continuity between the Pedicel and Endosperm.

Longitudinal sections of 12- to 16-DAP kernels representing the basal endosperm and pedicel are shown. There is no gap between the endosperm and the pedicel in genotypes with three and two copies of the *mn1-89* allele shown in **(A)** and **(B)**, respectively; however, the reciprocal hybrid with a single copy of the *mn1-89* allele shown in **(C)** developed a gap similar to that seen in the homozygous *mn1-1* parent shown in **(D)**.
(A) *mn1-89/mn1-89/mn1-89* homozygote.
(B) *mn1-89/mn1-89/mn1-1* hybrid.
(C) *mn1-89/mn1-1/mn1-1* hybrid.
(D) *mn1-1/mn1-1/mn1-1* homozygote.
Bar in **(A)** = 65 μm; em, embryo; en, endosperm; g, gap; p, pedicel.

Sh-encoded enzyme sucrose synthase (Chourey and Schwartz, 1971; Chourey and Nelson, 1979).

Additional genetic evidence is based on the nearly linear increases in invertase activity as well as on the CWI protein levels seen with increases in gene–dose levels at the *Mn1* locus. Because endosperm is a triploid tissue receiving two genes from the female parent and one from the male parent, it was possible to obtain endosperm genotypes with zero, one, two, and three copies of the derived allele and to correlate these with levels of the corresponding enzyme activity or protein. Three sets of hybrids, *Mn1/mn1-1*, *Mn1/mn1-89*, and *mn1-89/mn1-1*, of lineage-related parents were analyzed, and a gene–dose relationship of enzyme activity and the protein was observed for each set. Several loci with well-established

Figure 3. (continued).

The cell wall invertase protein was predominantly localized to wall ingrowths and the apoplastic region in basal endosperm cells in *Mn1* kernels.
(A) *Mn1/Mn1/Mn1*, treated with preimmune serum.
(B) to **(D)** *Mn1/Mn1/Mn1.*
(E) *mn1-89/mn1-89/mn1-89.*
(F) *mn1-1/mn1-1/mn1-1.*
(G) *mn2/mn2/mn2.*
Bars = 65 μm in **(A)**; 13 μm in **(C)**; 2.6 μm in **(D)**. The scale in **(B)** and **(E)** to **(G)** is the same as in **(A)**. em, embryo; en, endosperm; g, gap; p, pedicel.

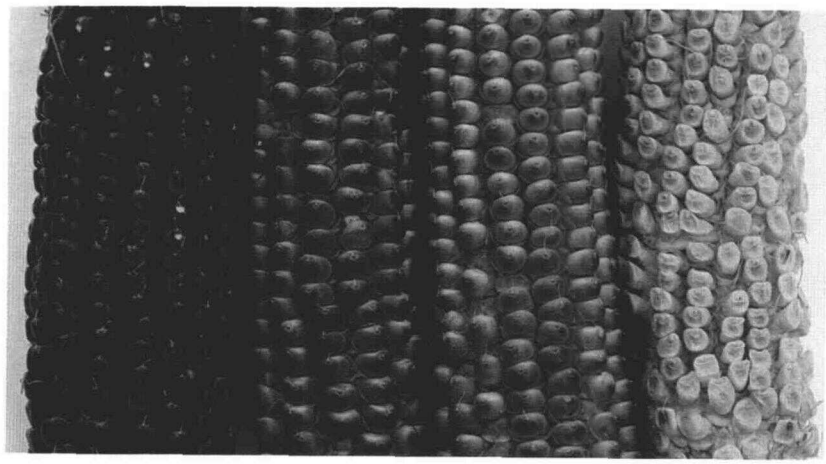

Figure 5. Variability in Seed Phenotypes Is Dependent on the Copy Number of the *mn1-89* Allele.

Endosperm genotypes from left to right are *Mn1/Mn1/Mn1*, *mn1-89/mn1-89/mn1-89*, *mn1-89/mn1-89/mn1-1*, and *mn1-89/mn1-1/mn1-1*.

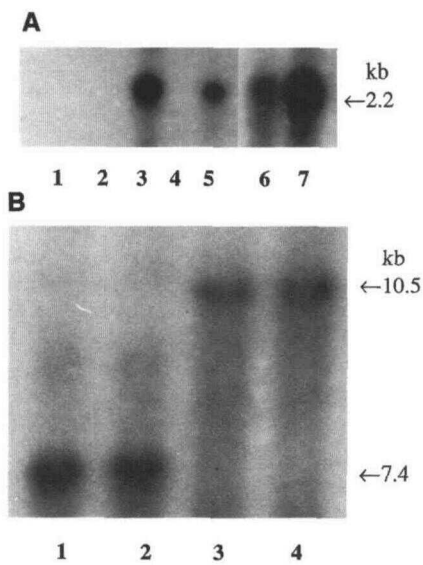

Figure 6. The *incw2* cDNA Clone Discriminates between *Mn1* and *mn1* Alleles on RNA and DNA Gel Blots.

(A) Gel blot with 1 μg of poly(A)$^+$ RNA in each lane from a maize cell suspension culture (lane 1) and 12-DAP kernels of *mn1-1* (lane 2), *mn1-74* (lane 3), *mn1-88* (lane 4), *mn1-84* (lane 5), *mn1-89* (lane 6), and *Mn1* (lane 7). Seed phenotypes for genotypes in lanes 3 to 5 were indistinguishable from *mn1-1* (lane 2). Consequently, the RNA in these genotypes may represent a pooled sample of kernels segregating for the homozygous genotype *mn1-1*, as shown, and the heterozygotes between the two parents.

(B) Detection of BamHI fragments in 10 μg of DNA in each lane from individual seedlings of *Mn1* (lane 1), *mn1-89* (lane 2), *mn1-1* (lane 3), and *mn1-1* (lane 4). Lanes 1 to 3 are in the lineage-related W22 inbred background, and lane 4 is of unknown background.

Numbers at right indicate molecular lengths of RNA in **(A)** or DNA in **(B)** in kilobase pairs.

gene–enzyme relationships have also shown similar linearity with their gene products (Nelson and Pan, 1995).

Finally, the molecular evidence, in particular our RNA gel blot data, showed that the *incw2* clone further subdivided the five EMS *mn1* mutants (which were all null at the protein level) into two groups. The first group showed detectable levels of the *incw2* transcripts, and the other group lacked such RNA, as was also the case with the *mn1-1* allele. The sixth mutant, *mn1-89*, was the only mutant in which RNA levels of expression were also associated with detectable levels of the CWI-2 protein, albeit at much reduced levels as compared with the wild type. Although a detailed characterization of these mutants remains to be done to elucidate the molecular basis of their expression, such a division of the null alleles into two classes is strong evidence that the *incw2* RNA is the encoded product of the *Mn1* gene. An alternative hypothesis is that the *Mn1* locus encodes an unknown factor, for example, a transcription factor, that controls the invertase gene at the RNA level of expression. This is highly unlikely. In this scenario, not only would such a factor need to discriminate various *mn1* alleles but would also have to exert post-transcriptional control over the *mn1-89* allele such that gene expression at the RNA level would not be correlated with the protein level of expression. To the best of our knowledge, such allelic discrimination in transregulation is currently not described. In contrast, such allelic diversity in gene expression at the RNA level of expression in the encoded gene product is a common occurrence. Additional support for our hypothesis is from both the restriction fragment length polymorphism and cosegregation mapping data that place *incw2* on the short arm of chromosome 2, close to the *mn1* locus, which itself remains to be mapped precisely. Although our cosegregation population was extremely limited in size, it is noteworthy that the same-sized BamHI fragment was seen both in the parental mutagenized allele, *Mn1*, and in the *mn1-89* mutant. EMS is a powerful mutagen that induces

point mutations in general (Aukerman et al., 1991; Bartel and Fink, 1995), and it is not likely to show genomic rearrangement, as is the case with insertional elements.

Possible Basis for Coordinate Control of Cell Wall-Bound and Soluble Forms of Invertase Activities

Our analyses of invertase activity—in particular, the cell wall–bound form and the soluble form—at various developmental stages (Figure 1) as well as in various genotypes (Table 1) have led to an entirely unexpected observation that the two isozymes are under a coordinate-like control. In each case, increases and decreases in the levels of the predominant cell wall–bound form were always associated with a similar correlated change in the soluble form. A similar pattern of these two isozymes has also been noted in a limited sample of genotypes (Miller and Chourey, 1992) and has been attributed to a possible common genetic basis for the two isoenzymes, as is the case in yeast (Carlson and Botstein, 1982). However, this hypothesis is now ruled out by more recent data showing that these two isozymes are clearly encoded by two distinct genes in various plant species (Unger et al., 1994; Weil et al., 1994; Greiner et al., 1995; Roitsch et al., 1995; Weber et al., 1995), including maize (Shanker et al., 1995; Xu et al., 1995).

It is noteworthy that an antisense invertase gene in transgenic tomato effectively suppressed both cell wall and soluble invertase enzyme activities (Ohyama et al., 1995); however, these authors did not elaborate or suggest a possible basis for this phenomenon. Similarly, Cheikh and Jones (1995) have also reported correlated changes in both forms of invertases in developing maize kernels after heat stress. Assuming a two-gene control for these two forms of invertases in the developing endosperm, we suggest here that invertases may also belong to a growing list of enzymes that are regulated by metabolic controls mediated by the sugars (for reviews, see Sheen, 1994; Thomas and Rodriguez, 1994). Indeed, Roitsch et al. (1995) have already described a certain level of metabolic control of invertase by sugars in cell suspension culture of *Chenopodium rubrum*. It is probable that the unique spatial distribution of invertase in maize endosperm, with the highest level at the base and undetectable levels in the upper region, is reflective of, among other factors, the decreasing gradient of sucrose concentration, which is highest at the base and lowest at the tip (Doehlert and Kuo, 1990). Although there are nearly equal levels of ^{14}C-sucrose in *Mn1* and *mn1* endosperms following a $^{14}CO_2$ pulse to plants of these two genotypes (Shannon et al., 1993), a lack of sucrose hydrolysis in the *mn1* endosperm apoplast must "lock-out" sugars from entering the cytosol and may lead to significant down-regulation of the intracellular soluble invertase. Consistent with this speculation are our data from maize cell suspension culture showing that depletion of sucrose from culture medium led to a time-dependent coordinated loss of both cell wall and soluble forms of invertase activities (W.-H. Cheng and P.S. Chourey, unpublished data). The specific details on how metabolic status of sugars modulates expression of various genes, including invertases, remains to be elucidated.

A Critical Role of Invertase in Stability of Maternal Cell and Sink Strength in Endosperm

The earliest detectable event of the *mn1* seed trait anatomically is the withdrawal of pedicel from developing endosperm (i.e., a gap formation) at ~9 to 10 DAP (Lowe and Nelson, 1946). Our interpretation of the collective data shown in Figure 4 and Table 1 is that the normal attachment, or the early withdrawal, of the pedicel from endosperm is controlled in a rate-limiting manner by the levels of invertase activity in the endosperm. These results are in full agreement with, and in fact have extended further, our previous postulate that the stability of placento-chalazal cells in the pedicel is determined by the endosperm (Miller and Chourey, 1992). The current data (Table 1 and Figure 4) are important for two reasons. First, when compared with the previous value of ~20% of wild-type enzyme activity in the *mn1/Mn1* hybrid (Miller and Chourey, 1992), a significantly lower level of invertase activity, only ~4 to 6% that of the wild type, was sufficient to arrest the gap formation in *mn1-89/mn1-1* hybrids. Second, the reciprocal hybrid with a single copy of the *mn1-89* allele and only ~2% wild-type levels of invertase activity was distinctly marked by a gap formation in the pedicel–endosperm region, as was also seen with the *mn1-1* homozygote. Thus, the correlated changes, anatomical as well as biochemical, in lineage-related genotypes, constitute unequivocal evidence that the invertase-dependent metabolic status of endosperm affected the stability of cells in the pedicel.

As a possible basis for such an interdependence of the pedicel on endosperm, we have previously suggested that invertase deficiency in *mn1-1* endosperm may lead to a transient accumulation of sucrose and osmotic imbalance in this region and, consequently, the degeneration of ultrathin placento-chalazal cells in the pedicel (Miller and Chourey, 1992). This hypothesis is now considerably strengthened because Shannon et al. (1993) have indeed observed elevated levels of ^{14}C-sucrose in the pedicel of *mn1-1* relative to *Mn1*, following a $^{14}CO_2$ pulse. To the best of our knowledge, this is probably the most direct demonstration that CWI plays a crucial role in osmoregulation and/or turgor sensing in sugar transport, as has been suggested previously based mainly on inferential results (von Schaewen et al., 1990; Ramloch-Lorenz et al., 1993; Weber et al., 1995). The role of the CWI protein seen in the pedicel (p68) is presently unclear and is under investigation. Based on the difference in its size relative to the CWI-2 subunit (p72), we believe it is probably independent of the *Mn1* locus.

Because invertases are spatially and temporally the first enzymes in developing endosperm to metabolize the incoming sucrose, it is not surprising that the loss of such activity, as

in the *mn1-1* mutant, is associated with a significant reduction in mature seed weight. In fact, our data for the *mn1-89* mutant and its hybrids with the *mn1-1* parent showed a dramatic rate-limiting effect of this enzyme on sink strength of a developing endosperm, as evidenced from the increases in seed weights. Overall, these data also provide a metabolic basis for the semi-dominant nature of the *mn1-89* allele over *mn1-1*, because seed phenotype in the hybrid was dependent on the levels of endosperm invertase activity. However, it is important to note that such a relationship was limited to only a narrow range of activity; a 6% level of the wild type was sufficient to yield a near–wild type phenotype (~77% of the wild-type seed weight). It is reasonable to suggest that a genotype with only a slight increase, perhaps ~8 to 10% of the wild-type levels of enzyme activity, would be sufficient to obtain a wild-type (*Mn1*) seed phenotype. Clearly, a large proportion of the enzyme, ~90% of the wild-type level, was dispensable without a significant change in seed phenotype. A large excess of enzyme activity appears to be a common feature with many enzyme systems in diverse plant species (Chourey and Nelson, 1979; Quick et al., 1991; Zrenner et al., 1993).

In conclusion, these data provide strong evidence for a critical role of the *Mn1*-encoded CWI-2 enzyme in the appropriate partitioning of sucrose in a developing maize endosperm. The CWI-2 enzyme is spatially and temporally the first enzyme in the endosperm to metabolize the incoming sucrose from the plant. Shannon et al. (1993) have shown nearly equal levels of ^{14}C-sucrose in *Mn1* and *mn1-1* endosperms after a $^{14}CO_2$ pulse to the plants. Obviously, the CWI-2–based cleavage of sucrose in the basal endosperm cells is a critical step that determines the unidirectional flux of carbon entering the downstream reactions in the endosperm. There is also correlative evidence that sucrose is resynthesized in this part of the endosperm (Shannon et al., 1986; Chourey et al., 1995). Thus, a cycle of sucrose cleavage and resynthesis, similar to the "futile" cycle described in germinating cotyledons of *Ricinus communis* (Geigenberger and Stitt, 1991), could contribute to a regulatory force in maintaining the physiological gradient between phloem termini in the pedicel and the developing endosperm.

METHODS

Isolation of *miniature1* Seed Mutants by Ethyl Methanesulfonate

Almost 20,000 maize (*Zea mays*) seed homozygous for *Miniature1* (*Mn1*) and anthocyanin genes in the W22 inbred background were soaked in 0.08 M aqueous solution of ethyl methanesulfonate (EMS; Sigma) for 10 hr in a fume hood at room temperature. When EMS treatment was completed, the solution was decanted and the seed were washed in running water for 24 hr. Seed were later spread as a thin layer on paper towels to dry for ~2 weeks at 8°C in a walk-in cold room (Briggs et al., 1965; Chourey and Schwartz, 1971).

Treated seed were planted and used as female parents in crosses with homozygous *mn1-1* tester stocks (colorless seed). Screening for mutants with the *mn1* seed phenotype in a population of 1200 F_1 ears led to seven ears that showed a variable number of mutant kernels along with the wild-type seed on each ear. Each F_1 ear with mutant kernels was considered to be an independent mutant allele, and all mutant kernels on the same ear were treated as originating from the same mutational event. Five of the seven ears had mutant kernels that were indistinguishable from the *mn1-1* seed phenotype, whereas the mutant kernels on two ears were only slightly reduced in seed size as compared with the wild type. Anthocyanin pigment in mutant seed was also slightly reduced as compared with their counterpart wild-type seed on each of the seven ears. When selfed, F_2 ears from the five new mutants, *mn1-74*, *mn1-82*, *mn1-83*, *mn1-84*, and *mn1-88*, showed kernels of only the *mn1-1* seed phenotype. Consequently, it was not possible to identify the homozygous kernels of the new mutant allele relative to the *mn1-1* used as the male parent; moreover, there were no known linked markers associated with the mutagenized *Mn1* allele. However, two of the mutants, *mn1-87* and *mn1-89*, were readily identifiable in the F_2 generation due to their unique seed phenotypes. The detailed analyses on *mn1-87* are currently in progress. All plants were grown in the field and/or greenhouse with normal diurnal (light/night) conditions. After hand pollinating, the developing seeds were harvested at various developmental stages and stored in −80°C until use.

Genetic Crosses for Determining Seed Weights

F_2 ears were obtained by selfing the *Mn1/mn1-1* and *Mn1/mn1-89* F_1 heterozygotes. Several F_2 ears showing a 3:1 segregation for the wild type and either the homozygous recessive *mn1-1* or *mn1-89* kernels were obtained. Homozygous mutant kernels from several F_2 ears were pooled and weighted against the corresponding *Mn1* kernels, representing one, two, and three copies of the *Mn1* allele. Similarly, two sets of testcrosses with (1) *Mn1/mn1-89* × *mn1-1/mn1-1* (male parent) and (2) *Mn1/mn1-1* × *mn1-89/mn1-89* (male parent) were made. Both sets led to BC1 ears with seeds of two distinct seed sizes that segregated in a 1:1 ratio. Based on our previous genetic analyses with these genotypes, we attributed the smaller seeds to endosperm genotypes as *mn1-89/mn1-89/mn1-1* and *mn1-89/mn1-1/mn1-1* in set (1) and set (2), respectively. The larger seeds in each set were due to the dominant *Mn1* allele.

Invertase Enzyme Assays

Frozen kernels were homogenized in extraction buffer in a 1:10 (w/v) ratio by using a cooled mortar and pestle. The extraction buffer used in the isolation of soluble invertase protein contained 50 mM Tris-maleate, pH 7.0, and 1 mM DTT (Doehlert and Felker, 1987). The homogenate was centrifuged at 14,000*g* for 10 min; the supernatant was removed for soluble invertase assays followed by 1-hr or overnight dialysis. The pellet was washed three times in extraction buffer followed by a final resuspension in extraction buffer containing 1 M NaCl in a 1:2 (w/v) ratio. The salt suspension was vortexed in an Eppendorf mixer (model 5432; Eppendorf Corp., Madison, WI) for 30 min at 3°C and subsequently centrifuged at 14,000*g* for 10 min. The supernatant was dialyzed against extraction buffer without NaCl at 3°C for 1 hr or overnight and used as cell wall–bound invertase fraction in enzyme assays, as described previously (Tsai et al., 1970; Miller and Chourey,

1992). Because there is no detectable level of invertase activity in developing embryos (Doehlert and Felker, 1987; Miller and Chourey, 1992), the terms endosperm and kernel are used interchangeably in this study.

Protein Gel Blot Analysis

Denatured protein samples were separated on an SDS–polyacryamide gel, according to Laemmli (1970). For gel blot analyses, proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) and treated according to the instructions provided with a Du Pont staining kit (New Renaissance). Briefly, the membrane was blocked in 5% nonfat dry milk in 10 mM PBS-T (PBS–Tween 20) for 1 hr. After two washes with PBS-T, each for 5 min, the membrane was incubated with polyclonal antibodies raised against carrot cell wall–bound invertase (CWI) for 1 to 2 hr. The carrot antibody was a kind gift from A. Sturm (FMI, Basel, Switzerland). After the first wash for 15 min and four washes, each for 5 min, the membrane was incubated with the secondary antibody anti-rabbit immunoglobulin conjugated with horseradish phosphatase (Sigma) for 1 hr. After four washes, the membrane was treated with chemiluminescence reagent (Nel-100; Du Pont) for 1 min before developing.

Immunohistochemical Localization

Developing kernels at various stages were immediately fixed in formalin acetic alcohol, dehydrated through tertiary butyl alcohol series, infiltrated in Paraplus (Fisher Scientific), and embedded and immunostained essentially following the protocol described by Chen and Chourey (1989), with the few exceptions noted below. In brief, to ensure the complete removal of tertiary butyl alcohol from infiltrated kernels, they were subjected to three changes of fresh liquid Paraplus, each for 2 to 3 hr, before embedding. Cross-sections were cut ~10 μm in thickness by using a rotary microtome. Paraffin was removed from sections on the slide in xylene and sequentially hydrated to 30% ethanol. After further washing in distilled water and PBS, slides were incubated with primary antibody or preimmune serum as a negative control for ~4 hr. The slides were then incubated with secondary antibody solution composed of biotinylated anti–mouse anti-rabbit immunoglobulin and streptavidin alkaline phosphatase. Immunolocalized signal of CWI was visualized using New Fuchsia chromogen (Dako Corp., Carpinteria, CA), resulting in a precipitate of fuchsia-colored end product at the site of the antigen.

DNA and RNA Blot Analyses

For RNA gel blots, total RNA was isolated from 12-days-after-pollination (DAP) kernels and maize cell suspension culture, as described previously (Wadsworth et al., 1988). Poly(A)$^+$ RNA was isolated according to Ausubel et al. (1995). The RNA was glyoxalated and fractionated on a 1.2% agarose gel. RNA was transferred to a Nytran membrane (Schleicher & Schuell) and prehybridized in 50 mM Pipes, pH 6.5, 100 mM NaCl, 50 mM sodium phosphate, pH 6.5, 1 mM EDTA, and 5% SDS. The blots were hybridized in the same solution with 3 × 10^6 cpm/mL of solution overnight at 65°C. Blots were rinsed two times for 45 min in 6 × SSC (1 × 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 two times for 30 min in 0.2 × SSC, 5 mM EDTA, pH 8.0, 5 mM sodium phosphate, pH 6.5, 1% SDS. The blots were exposed to x-ray film for 4 days with two intensifying screens.

Genomic DNA was isolated from 7-day-old dark-grown seedlings, as described by Dellaporta et al. (1983). DNA (~10 μg) was digested with BamHI, according to the manufacturer's specifications (Gibco BRL, Gaithersburg, MD). The digested samples were fractionated on 0.6% agarose gels, transferred to a Nytran membrane, and hybridized with the ^{32}P-labeled ~2.2-kb cDNA probe *incw2*. Gel blot hybridization and washes were performed as described previously (Gupta et al., 1988). Briefly, membranes were hybridized in the same manner as were the RNA gel blots (see above).

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