The Plant Cell, Vol. 4, 297–305, March 1992 © 1992 American Society of Plant Physiologists

The Maize Invertase-Deficient *miniature-1* Seed Mutation Is Associated with Aberrant Pedicel and Endosperm Development

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Genetic evidence is presented to show that the developmental stability of maternal cells in the pedicel at the base of maize seeds is determined by the genotype of the developing endosperm. An early degeneration and withdrawal of maternal cells from the endosperm of homozygous *miniature* (*mn mn*) seed mutants were arrested if *mn* plants were pollinated by the wild-type *Mn* pollen. Similarly, the stability of the wild-type, *Mn mn*, maternal cells was also dependent on whether or not these cells were associated with the normal (*Mn*) or the mutant (*mn*) endosperm on the same ear. Biochemical and cellular analyses indicated that developing *mn* kernels have extremely low (<0.5% of the wild type) to undetectable levels of both soluble and wall-bound invertase activities. Extracts from endosperm with a single copy of the *Mn* gene showed a significant increase in both forms of invertases, and we suggest it is the causal basis of the wild-type seed phenotype. Collectively, these data provide evidence that invertase-mediated maintenance of a physiological gradient of photosynthate between pedicel and endosperm constitutes the rate-limiting step in structural stability of maternal cells as well as normal development of endosperm and seed.

INTRODUCTION

The normal development and growth of seeds in higher plants are significantly dependent on the maternal diploid tissue at the base of the seed, the pedicel. Photosynthate and nutrients are unloaded into the pedicel through vascular elements prior to their entry into the basal endosperm cells and subsequent mobilization into the upper parts of endosperm and embryo. The lack of plasmodesmatal connections between pedicel and endosperm (Felker and Shannon, 1980; Thorne, 1985) excludes the symplastic entry of mobile elements such as viruses into the seed. Despite the major role of the pedicel region, little is known concerning molecular and physiological aspects of pedicel-specific gene expression and possible interactions between the pedicel and the filial generation. Similarly, the transfer cells at the base of the endosperm in maize, as in all cereals, are anatomically and functionally different from the rest of the endosperm (Kiesselbach, 1949; Schel et al., 1984; Shannon et al., 1986; Chen and Chourey, 1989). Although there is a significant body of biochemical and molecular genetic knowledge on genes specific to the upper part of the endosperm that are engaged primarily in storage function, very little is known about genes specific to the lower part of the endosperm. The present report focuses on developmental and biochemical genetic aspects of the *miniature* (*mn*) seed mutant in maize, in which the earliest manifestation of mutant expression is in the pedicel and the basal part of the endosperm.

The *mn* seed mutant was first described by Lowe and Nelson (1946). The homozygous recessive *mn* mutant phenotype is specific to the seed; it is only one-fifth the weight of normal (*Mn*) seeds and exhibits single gene inheritance. Histological studies show that the development of *mn* kernels is normal up to 9 days after pollination; soon thereafter there is a withdrawal of maternal cells, and a gap occurs between the pedicel and the basal region of the endosperm. The loss of the chalazal bridge in *mn* kernels leads to near arrest of endosperm and embryo development at ~14 days after pollination. Lowe and Nelson (1946) speculated that the *mn* endosperm is lacking a substance that is critical to the conducting cells. In barley, similar mutants affected in the pedicel region showed maternal inheritance (Felker et al., 1985). We have analyzed the *mn* seed mutant and describe the following features: (1) Despite the early manifestation of the *mn* gene in maternal cells, the mutant trait was not inherited maternally but as an endosperm trait (i.e., showed xenia effect). (2) The developing *mn* kernels were deficient in invertase activity in both maternal and endosperm cells. (3) Genetic evidence indicated that invertase activity in basal endosperm cells directly affected the metabolic and developmental stability of the maternal cells. Thus,

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a metabolic deficiency constituted the basis for a unique interaction between the cells of the two generations within the seed.

RESULTS

The *mn* Seed Is Inherited as an Endosperm Trait

Because the earliest detectable anatomical lesion of the *mn* seed mutant is in the pedicel region (Lowe and Nelson, 1946; see Figure 1), it was of significant interest to test whether this trait is maternally inherited. Reciprocal crosses were made between plants of homozygous wild-type and mutant genotypes. We observed the wild-type seed phenotype independent of the mode of cross, indicating that *mn* seed is inherited as an endosperm trait and is not maternally inherited. Test crosses with an *Mn mn* heterozygote as the female parent (contributing to the wild-type pedicel) and a homozygous recessive mutant as the male parent were also made. Such crosses yielded a segregation of 706:743 of wild-type and mutant kernels, respectively, from a total of eight ears. These numbers are in agreement with a 1:1 segregation ratio, further supporting the previous observation that *mn* seed is an endosperm trait, even though the earliest manifestation of the anatomical abnormality is in the maternal cells.

Figure 1 shows scanning electron micrographs of longitudinal sections of kernels of homozygous wild-type and recessive genotypes at 12 days after pollination and the hybrid derived from the cross between mutant as female and homozygous wild type as the male parent. In the wild-type genotype (Figure 1A), the uppermost layer of the pedicel, placentochalazal cells, was in close contact with the developing endosperm. The corresponding region in the mutant kernel (Figure 1B), at the same developmental stage, was withdrawn from the endosperm, as first demonstrated by Lowe and Nelson (1946). The anatomical abnormality in the mutant seed is restricted to maternal cells in the pedicel; i.e., no detectable change was seen in the endosperm. Similar sections derived from F_1 hybrid kernels (Figure 1C), representing *mn mn* and *Mn mn mn* genotypes of pedicel and endosperm, respectively, showed no detectable differences as compared to the homozygous wild-type parent. There was no withdrawal of maternal cells from the hybrid endosperm, unlike with the *mn* seed mutant. Test cross ears (i.e., *Mn mn* as female × *mn mn*) yielded wild-type and mutant kernels on the same ear and were readily identifiable at 12 days after pollination by their size differences. Scanning electron microscopy (SEM) analyses of several kernel sections showed a clear withdrawal of pedicel from endosperm in the *mn* type, but not in *Mn* seeds (data not shown). Thus, clearly the endosperm genotype determines whether the wild-type, *Mn mn*, pedicel will be attached or withdrawn from the endosperm.

The *mn* Mutant Is Deficient for Invertase in Developing Seed

We used a histochemical stain (Doehlert and Felker, 1987) to localize invertase enzyme activity in longitudinal free-hand sections of kernels 12 days after pollination of the wild type and the *mn* mutant. Figure 2 illustrates the results of the assay; abundant levels of enzyme activity, as judged by the sucrose-dependent black stain caused by formazan reaction, were seen in the wild type (Figure 2B) but not in the mutant genotype (Figure 2C). The enzyme activity was detectable in both the pedicel at the base and the lower part of the endosperm, as described previously (Doehlert and Felker, 1987). It was of interest that no activity was detected in either the pedicel or the

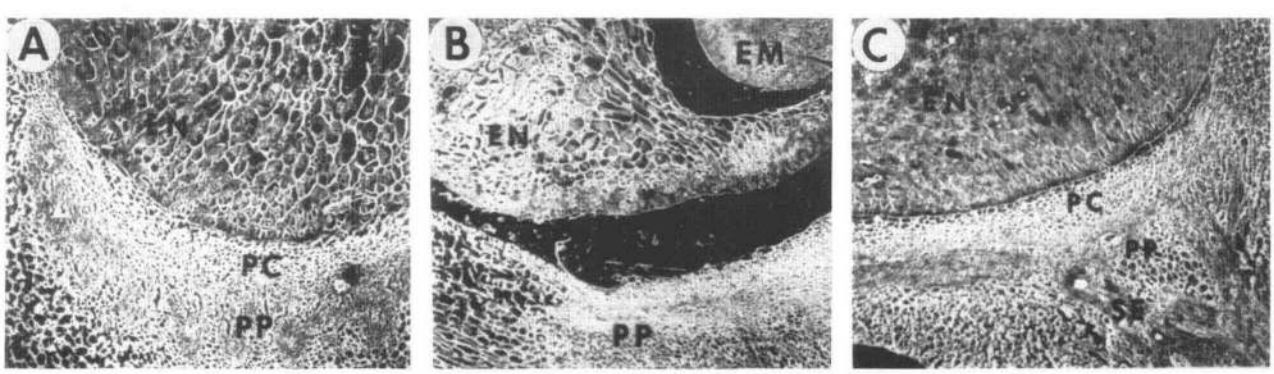

Figure 1. Scanning Electron Micrographs of Basal Portion of Maize Kernels at 12 Days after Pollination.

(A) Homozygous *Mn Mn* genotype. The placentochalazal region in the pedicel is in close contact with the lower portion of endosperm. ×36.
(B) Homozygous *mn mn* seed mutant. The placentochalazal region is withdrawn from the homozygous recessive endosperm. Gap formation between the two tissue results from cell rupture and degeneration and restricts the flow of photosynthate to the developing kernel. ×35.
(C) Heterozygote obtained by crossing an *mn mn* female × *Mn mn* male. The *mn mn* pedicel is in close contact with *Mn mn mn* endosperm. ×35.
PC, placentochalazal; EN, endosperm; PP, pedicel parenchyma; EM, embryo; SE, sieve elements of phloem.

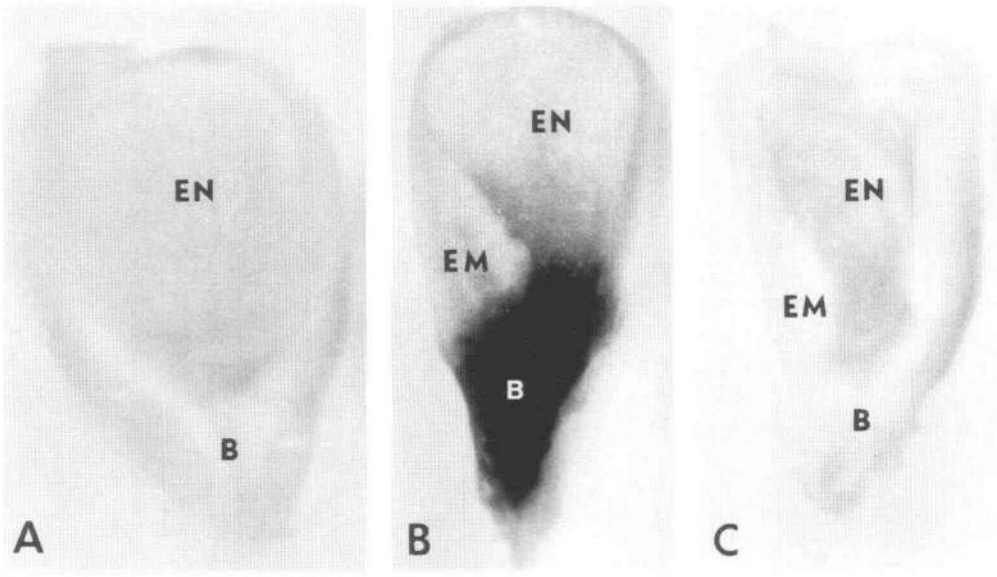

Figure 2. Histochemical Localization of Invertase Activity in Kernels 12 Days after Pollination.

(A) Homozygous *Mn Mn* kernel section incubated without sucrose, a positive control for detection of invertase activity.
(B) Homozygous *Mn Mn* kernel section incubated with sucrose demonstrating sucrose-dependent localization of invertase activity in the basal portion of the kernel that includes both lower endosperm and pedicel. Embryo showed no detectable levels of invertase activity.
(C) Homozygous *mn mn* kernel section incubated with sucrose showing lack of invertase activity in the basal part of the kernel.
B, basal portion of the kernel including lower endosperm and pedicel tissue; EN, endosperm; EM, embryo.

endosperm in *mn* sections. Many individual kernels of the wild-type and the mutant phenotype on segregating F_2 ears were also examined histochemically; invertase deficiency segregated exclusively with the *mn* phenotype. Similar tests were done on kernel sections of several nonallelic starch-deficient endosperm mutants, including *shrunken-1*, *shrunken-2*, *shrunken-4*, *shrunken-5*, *brittle-1*, *brittle-2*, and *defective endosperm–1*. All of these mutants showed a positive reaction for invertase activity (data not shown), as seen in the wild type (Figure 2B). The invertase deficiency was specific to the *mn* seed mutant.

More detailed analyses on the wild type and *mn* mutant were done using crude extracts prepared from the lower third of the endosperm, approximately overlapping the black-stained region seen by histochemical tests. We assayed only the lower part of the endosperm because initial tests on the upper two-thirds of the endosperm showed undetectable levels of invertase activity. Spectrophotometric determinations of enzyme activity were done for both soluble and cell wall-bound forms of invertases using endosperm from 12 and 16 days after pollination. A previous study (Tsai et al., 1970) showed that invertase activity in maize endosperm peaks at 12 days after pollination. However, in this study, all three genotypes with the wild-type seed phenotype exhibited the highest invertase specific activity at 16 days after pollination, as shown in Table 1. By 20 days after pollination, there was an abrupt drop in activity; genotypes with three, two, and one copy of *Mn* genes showed specific activity values of 9.3, 10.7, and 1.9, respectively. The trace level of activity in the homozygous recessive mutant at 12 days after pollination was consistently seen in

Table 1. Comparison of Invertase Activity Levels as a Function of the Number of *Mn* Gene Copies in Endosperm

	12 DAP			16 DAP		
Endosperm Genotype	Soluble	Bound	Total	Soluble	Bound	Total
Mn Mn Mn	2.0	21.6	23.6 (100%)	1.1	29.0	30.1 (100%)
Mn Mn mn	1.8	20.0	21.8 (92%)	4.6	35.7	40.3 (134%)
Mn mn mn	0.4	3.7	4.1 (17%)	1.2	7.5	8.7 (29%)
mn mn mn	0.01	0.07	0.08 (0.33%)	0	0	0 (0%)

Specific activity values are expressed as micromoles per liter of reducing sugars per milligram of protein per minute and represent replicated assays. Values within parentheses are normalized to the homozygous *Mn* genotype. DAP, days after pollination.

extracts from several ears harvested in different crops; the genetic basis of this enzyme is at present not known. An earlier developmental stage, 8 days after pollination, was also examined for the two homozygous genotypes. At this stage, maternal nucellus tissue constituted the bulk of the kernel; crude extracts from entire kernels of homozygous *Mn* and *mn* genotypes showed values of 3.15 and 0.18, respectively. The specific activity of invertase in the excised nucellus tissue in each genotype was about 0.12; thus, nearly two-thirds of the total invertase activity in *mn mn* kernels at 8 days after pollination is attributable to the maternal nucellus tissue. It is noteworthy that the *mn* mutant is deficient for both soluble and the bound forms of the enzyme. The invertase deficiency of the mutant is highly specific, as there were no detectable differences in the amount of total soluble protein or in the activity of another sucrose-metabolizing enzyme, sucrose synthase. Moreover, there were no detectable differences in the proteins between the two homozygous genotypes by the criteria of nondenaturing or denaturing gel electrophoresis. Because of the known proteinaceous inhibitors of plant invertases (Bracho and Whitaker, 1990), appropriate in vitro mixing experiments (see Methods) between the mutant and the wild-type endosperm extracts were also conducted. These mixtures suffered no loss of enzyme activity (data not shown), indicating that the invertase deficiency of the *mn* mutant is not attributable to invertase inhibitors. Invertase activity in young roots of 4- to 5-day-old *mn* seedlings is unaffected; thus the alteration is kernel specific, as is usually the case with many kernel- or endosperm-specific mutants in maize.

Endosperm extracts from reciprocal hybrids containing one or two *Mn* gene copies were tested for the gene–dose relationship of invertase activity (Table 1). At 12 days after pollination, there was no difference in the level of enzyme activity between the genotypes with two and three copies of *Mn* genes. At 16 days after pollination, extracts from the *Mn Mn mn* genotype showed a much higher level of enzyme activity than the homozygote with three copies of the *Mn* gene. Clearly, there was no gene–dose relationship with the enzyme activity. There was, however, a marked increase of activity in both forms of invertase in the genotype with a single copy of the *Mn* gene relative to the homozygous recessive mutant. Although the increased level contributed to only ~17 and 29% at 12 and 16 days after pollination, respectively, relative to the homozygous wild type, it was sufficient to physiologically restore the mutant to a wild-type phenotype. It is worth noting that these invertase values, along with similar data on *shrunken-1* mutants deficient in sucrose synthase activity (Chourey and Nelson, 1979), suggest that most endosperm enzymes are present at much higher levels in vivo than the minimal threshold requirement for a wild-type phenotype.

Figure 3 shows further data regarding the cellular localization of enzyme activity in kernel sections of these four genotypes, particularly the *Mn mn mn* hybrid obtained from mutant plants (i.e., *mn* pedicel). As expected, the histochemical color intensity was much reduced in endosperm with a single copy of the *Mn* gene (Figure 3A) as compared to genotypes with two or three copies of the *Mn* gene. A remarkable feature is that the mutant pedicel region of the *Mn mn mn* kernel (Figure 3B) showed a readily detectable level of invertase activity; however, the mutant pedicel associated with the mutant endosperm, *mn mn mn*, yielded no detectable levels of invertase activity (Figure 3A). It is unlikely that the positive reaction in the pedicel of the *Mn mn mn* hybrid is due to diffusion of the stain from the endosperm for the following reasons. First, the formazan precipitate causing the black coloration is cell limited (Doehlert and Felker, 1987). Second, the stain reaction between the endosperm and pedicel in the hybrid is separated by a colorless layer of placentochalazal cells (Figure 3B). Thus, the low level of invertase activity in homozygous recessive maternal cells in hybrid kernels is attributable to a direct effect of the wild-type endosperm.

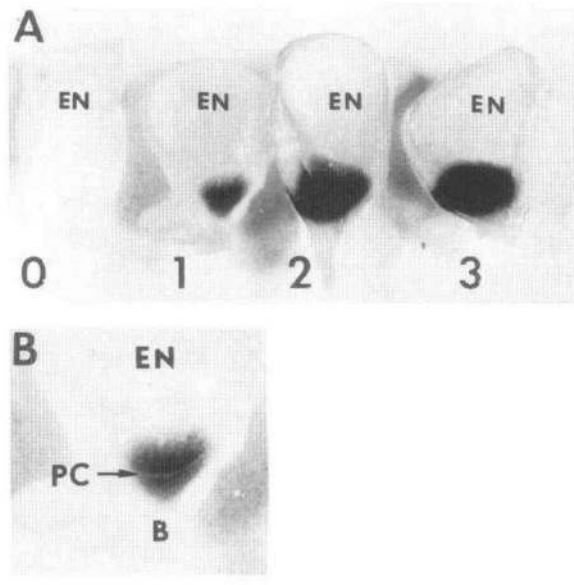

Figure 3. Histochemical Localization of Invertase Activity in Kernels 12 Days after Pollination Varying in Number of *Mn* Gene Copies.

(A) *Kernel designations 0, 1, 2, and 3 correspond to the number of Mn* gene copies within the endosperm. Hybrids were generated by reciprocal crosses between homozygous *Mn Mn* and *mn mn* parents. Kernel 0, the *mn mn* mutant, shows no detectable levels of invertase activity and possesses the characteristic *miniature* phenotype. Kernels 1, 2, and 3 show detectable levels of invertase activity in both lower endosperm and pedicel with normal kernel phenotypes.

(B) Enlarged view of kernel 1 (*Mn mn mn*) in **(A)** demonstrating a detectable level of invertase in both basal endosperm and maternal pedicel tissue separated by the nonstaining white layer corresponding to placentochalazal cells.

EN, endosperm; PC, placentochalazal; B, basal portion of pedicel.

Early Manifestation of *mn* Expression in Maternal Cells

A closer examination of cells associated with the pedicel and the basal endosperm region of the *Mn* as shown in Figure 4A and *mn* (Figures 4B to 4E) genotypes showed that the earliest anatomical alterations were localized to the pedicel region. We were able to observe the onset of separation between endosperm and pedicel in the *mn* mutant kernel as early as 7 days after pollination by both light microscopy and SEM. Figure 4B shows a scanning electron micrograph of a longitudinal section through an *mn* mutant kernel 7 days after pollination, revealing the commencement of cellular disruption in the maternally derived placentochalazal layer. At this early stage of lesion development in *mn* kernels, photosynthate may still be permitted access to and taken up by endosperm tissue. Maintenance of a sucrose concentration gradient, presumably mediated by the very low level of invertase at this stage, could be preserved through the placentochalazal cells that were not yet destroyed. Progression of cellular destruction increased as placentochalazal deterioration occurred. It should be pointed out that structural aberrations were restricted to placentochalazal cells; no changes were seen in the pedicel parenchyma cells. Figure 4B clearly shows unaltered phloem anastomosing throughout and terminating within thin-walled parenchyma cells of the pedicel. Pedicel parenchyma cells were persistent during the course of lesion development, and only those pedicel cells specifically designated as placentochalazal caused the gap to form.

By 12 days after pollination (Figures 4C and 4D), increasing losses of cellular integrity were apparent. Gap formation in the *mn* mutant was not simply the consequence of a passive withdrawal of pedicel away from endosperm tissue, but was associated with much cell rupture and degeneration. It was this premature separation of the pedicel-endosperm association that characterized the *mn* mutant phenotype anatomically. Closer examination (Figure 4E) of cells affiliated with placentochalazal tissue showed much cell wall degeneration, and remnants of broken cells were readily seen in the region normally marked by a physical contact between the pedicel and the endosperm. In sections of *mn* kernels, there was a clear indication that only maternally derived cells of the placentochalazal region were affected by this anatomical lesion. Endosperm cells in the immediate proximity to placentochalazal cells were structurally unaffected in the deterioration process. A tight interface was seen between pedicel and endosperm in similar sections from *Mn* kernels (Figure 4A). Lower endosperm cells are characterized by an elaborate system of cell wall ingrowths (Figure 4A). These specialized cells, collectively referred to as basal endosperm transfer cells, facilitate carbohydrate uptake. In all sections examined, we found no detectable structural aberrations of basal endosperm transfer cells in *mn* kernels (Figures 4C to 4E) as compared with the normal *Mn* kernels (Figure 4A). Complete severance of the pedicel-endosperm abutment served to finalize gap formation and arrested further development of endosperm and embryo in the *mn* seed.

It is perhaps possible to view gap formation within pedicel tissue as a processing artifact because pedicel parenchyma cells are extremely thin walled and would therefore be more prone to damage. However, in our investigation of other endosperm mutants and normal types as well, we found this particular phenomenon unique to only *mn* kernels. Thus, it is unlikely that the aberration is a processing artifact. As stated previously, the deterioration of maternal tissue was not a passive withdrawal or removal of pedicel from the endosperm tissue. Instead, the separation was viewed as the result of cell rupture and degeneration of cell wall components, as demonstrated histologically (Figures 4C to 4E).

We have recently analyzed another independently isolated *mn* mutant, mn-7690, both spectrophotometrically and histochemically for invertase activity. This mutant is indistinguishable from the *mn* reference allele, thus further supporting the data that invertase deficiency is associated with the *mn* mutation.

DISCUSSION

Sucrose is the principal and preferred form of photosynthate for long-distance transport to terminal storage sink tissue, such as the developing seed. Invertase is the first enzyme in the metabolic pathway that mobilizes photoassimilated sucrose into numerous reactions of the developing maize seed. Sucrose is unloaded through phloem termini in the pedicel, where the sucrose concentration is estimated to reach 400 to 500 mM (Shannon et al., 1986). Ample experimental evidence shows that there is a passive efflux of unhydrolyzed sucrose to basal endosperm cells (Porter et al., 1985, 1987), presumably due to large concentration differences between the pedicel and the endosperm. It was suggested that sucrose unloading may be regulated by pedicel cell turgor (Porter et al., 1987). The possibility of an energized sucrose/proton cotransport system was also suggested (Patrick, 1990); however, experimental evidence in maize endosperm is presently lacking. There is also evidence for apoplastic hydrolysis of sucrose in the pedicel region of the kernel prior to entrance in the endosperm (Doehlert and Felker, 1987). Unlike wheat caryopsis, in which sucrose is utilized without prior hydrolysis (Patrick et al., 1991), the majority of sucrose in the developing maize kernel is rapidly cleaved to glucose and fructose by invertases present in basal endosperm cells (Shannon, 1972; Doehlert and Felker, 1987). The results in Figure 2 and Table 1 further support this conclusion. Why maize has evolved such a complex and superfluous mode of transfer, relative to wheat, is not clear, except that there are several anatomical differences in the developing endosperm of the seed (Thorne, 1985). The hydrolyzed sucrose is subsequently mobilized to the upper part of the maize endosperm where the hexoses are partly resynthesized to sucrose prior to utilization in starch biosynthesis and numerous other metabolic functions (Shannon, 1972). We believe the data presented here collectively constitute strong genetic evidence

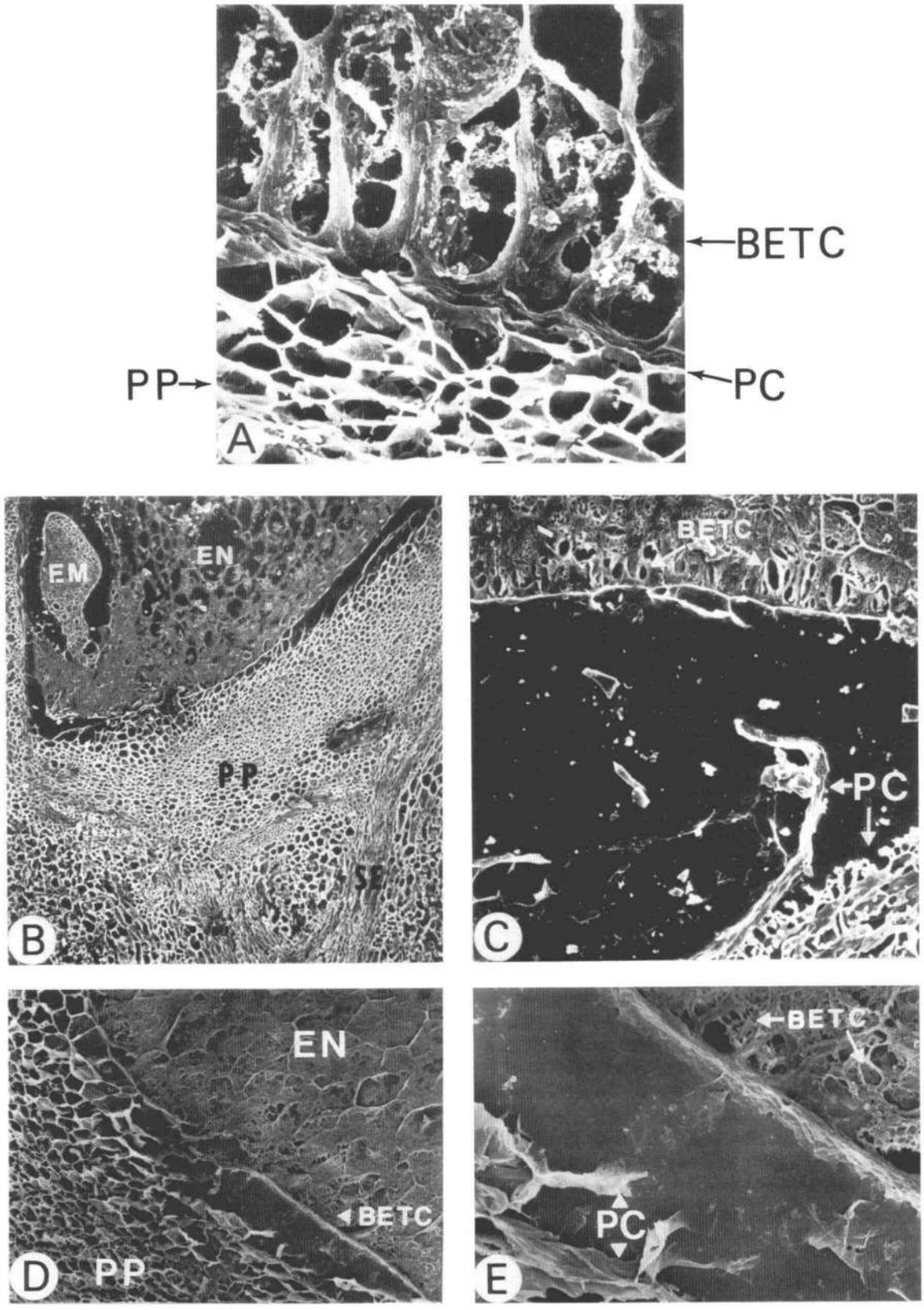

Figure 4. Scanning Electron Micrographs of Lower Portions of Wild-Type and *miniature* Mutant Kernels.

that invertases in basal endosperm cells exert a rate-limiting regulatory force in osmoregulation through a continuous pull of the photosynthate column from maternal cells to the cells of the filial generation. The invertase deficiency, as in the *mn* endosperm, gives rise to a multitude of events, notably in the pedicel region. These include cell degeneration mainly in the placentochalazal region, which is known to serve the function of absorption and transport of photosynthate and nutrients to endosperm and embryo (Schel et al., 1984). Cellular destruction results in gap formation between the pedicel and the endosperm. The loss of invertase in the *mn* mutant pedicel arises presumably from the absence of these cells. We infer that these cellular changes in the pedicel occur as a result of a transient osmotic imbalance due to the impaired movement of photosynthate to the endosperm.

Consistent with the conclusion that events in maternal cells are the result of endosperm invertases are the data from hybrid with the *Mn mn mn* genotype. A single copy of the *Mn* gene led to a marked increase in the levels of endosperm invertases and to a normal chalazal bridge as in the wild-type seed phenotype. Anatomically, the *mn* pedicel with the hybrid endosperm was, within our resolutional limits, indistinguishable from the *Mn* pedicel. However, the histochemical tests showed a marked reduction of detectable invertase in the pedicel region of *mn* as compared to the *Mn* kernel (Figure 3). This probably reflects the reduced sink strength of the endosperm (see below), which may feedback regulate the level of invertase activity in the pedicel. Alternatively, it is also possible that photosynthesis is sink limited (Oparka, 1990) and may reduce the density of assimilates passing through the pedicel. Further analyses based on in vitro kernel culture with various sugars are in progress to elucidate the possible basis for substrate regulation of invertases.

Invertases have been studied in many organisms, including plants. At least two forms of the enzyme, soluble and particulate, are a common feature to all organisms. The soluble form is predominantly localized to vacuoles and cytoplasm, and the particulate form, in all cases tested, is ionically bound to the cell wall and is readily extractable with high salt concentrations (Sturm and Chrispeels, 1990; von Schaewen et al., 1990; Weil and Rausch, 1990). The genetic basis of invertases and, in particular, the relationship between the two forms are unknown in plants. There are, however, elegant molecular genetic analyses in yeast to show that both forms of invertase are encoded by the single locus *sucrase 2* (Carlson and Botstein, 1982; Perlman and Halvorson, 1981). The *sucrase 2* gene encodes two different-sized transcripts that differ at their 5′ ends; the larger and the smaller RNAs code for glycosylated and soluble forms, respectively. Invertase-deficient strains, marked by a petite phenotype, show a loss of both forms of invertase (Carlson et al., 1981).

In plants, the maize *mn* seed mutant is perhaps unique in demonstrating invertase deficiency in the seed. It is noteworthy that, as in yeast, both soluble and bound forms are lost in the *mn* mutant. In addition, the endosperm genotype with a single copy of the *Mn* gene shows a marked increase in both forms of invertase as compared to the homozygous recessive mutant. These data are compatible with a hypothesis that the *Mn* gene codes for or modifies both soluble and bound invertases in the endosperm. An inconsistent feature, however, is the lack of gene–dose linearity, especially between the genotypes with two and three copies of the *Mn* gene, which showed near equal levels of enzyme activity in endosperm at 12 days after pollination. In addition, more enzyme activity was seen in the former than the latter at 16 days after pollination, which was quite unexpected. Previous studies on endosperm starch mutants in maize have shown gene–dose linearity with the levels of the corresponding enzyme activity (Hannah and Nelson, 1975; Chourey and Nelson, 1976). As an explanation, we suggest that invertase, which is a well-known biochemical marker of sink strength, is under more complex regulation than the enzymes engaged in starch biosynthesis. A hybrid endosperm with two *Mn* gene copies is likely to be a stronger sink due to numerous heterotic interactions than the homozygote with the three *Mn* copies. Although a similar argument can be made with the reciprocal hybrid, *Mn mn mn*, it is possible that sink strength in such a hybrid is severely rate limited due to the low level of gene expression from a single active *Mn* allele. Alternatively, we cannot formally rule out the unlikely possibility that endosperm invertase activities in these genotypes, including the homozygous *mn* mutant, is the effect of an unknown gene product encoded by the *Mn* locus. It is possible that *Mn* controls a function in endosperm that regulates the normal attachment between the endosperm and the pedicel.

Figure 4. (continued).

(A) Homozygous *Mn Mn* kernel at 12 days after pollination demonstrating the normal association between pedicel and endosperm. Note extensive wall ingrowth in basal endosperm transfer cells, perpendicular to the maternal cells including placentochalazal layer and pedicel parenchyma. ×630.
(B) Homozygous *mn mn* kernel at 7 days after pollination showing the commencement of cell degeneration and subsequent gap formation within the placentochalazal layer. ×63.
(C) Homozygous *mn mn* kernel at 16 days after pollination showing cell rupture and degeneration of placentochalazal layer. Basal endosperm transfer cells show no appreciable loss of structural integrity. ×170.
(D) Homozygous *mn mn* kernel at 12 days after pollination showing the involvement of placentochalazal cells in the degeneration process. ×250.
(E) Enlarged view of **(D)** providing closer detail of degeneration of placentochalazal cells. Remnant cell wall material can be seen where normal pedicel–endosperm contact should occur. Note that normal integrity of basal endosperm transfer cell, evidenced by extensive cell wall ingrowths, is maintained throughout the process of gap formation. ×1000.
BETC, basal endosperm transfer cell; PP, pedicel parenchyma; PC, placentochalazal; SE, sieve elements of phloem; EM, embryo; EN, endosperm.

The loss of this factor in *mn* seed leads to pedicel withdrawal and ultimately the impaired or much reduced flow of photosynthate to the endosperm. However, this does not account for widespread cell rupture in the *mn* pedicel region (Figure 4) or the significant reduction in endosperm invertases in the *Mn mn mn* genotype, where a normal chalazal bridge is maintained with the pedicel.

Finally, these data also bring to light an important feature of seed development—namely, that a metabolic step is intimately connected to developmental interactions between the endosperm and the maternal cells in the pedicel. Indeed, transgenic tobacco plants expressing excessive levels of yeast invertases also show significant developmental abnormalities in vegetative tissues (von Schaewen et al., 1990; Dickinson et al., 1991). We believe that the *mn* seed mutant with the impaired unloading process provides an ideal experimental system to analyze physiological aspects of the transfer of photoassimilates and nutrients from maternal cells to developing seed.

METHODS

Plant Material

Two successive selfed generations of the heterozygous *Mn mn* genotype yielded the homozygous dominant and recessive stocks that were grown in the field and/or greenhouse in three crops from 1990 to 1991. Plants were hand pollinated, and developing kernels were harvested at specific intervals appropriate to this particular study. Kernels destined for scanning electron microscopy (SEM) and histochemical analysis were processed at the time of harvest according to the procedures described below. Kernels used in enzyme assays were collected, immediately excised from the ear, frozen in liquid nitrogen, and stored at −20°C until use.

SEM

Immature mutant and normal maize kernels were harvested at various stages of development, identified by days after pollination. Because of the size and the starchy nature of the endosperm tissue, fixative penetration of kernels was facilitated by trimming the sides parallel to the embryonic axis. Kernels were subsequently fixed in formalin acid, dehydrated through an ethanol-tertiary butyl alcohol series, and infiltrated with Paraplast plus paraffin. After several changes of paraffin, the tissue was cast into blocks, and serial longitudinal sections were made 10 to 12 μm in thickness (Sass, 1945; Clark and Sheridan, 1986). Individual sections were placed onto glycerin/gelatin-coated 12-mm round coverslips, and the paraffin was removed with two changes of xylene and placed into 100% absolute ethanol. Upon removal of the sections from ethanol, each was covered with a drop of hexamethyldisilazane, which acts as a dehydrating agent replacing the more traditional critical point drying procedure. Sections were treated with hexamethyldisilazane in three successive applications with enough time between each application for complete air drying to occur. Following dehydration, the sections were cemented onto aluminum specimen support stubs, coated with gold by sputter coating, and examined in a scanning electron microscope (model No. 450; Hitachi Ltd., Tokyo, Japan).

Histochemical Staining

Histochemical staining methods for localizing invertase activity on maize kernel sections is based on a series of coupled redox reactions, which ultimately form a nonsoluble blue formazan precipitate. A modification of this reaction, described by Doehlert and Felker (1987), was utilized in this investigation. Briefly, immature mutant and normal kernels (12 days after pollination) were hand sectioned longitudinally and fixed in 4% formalin (pH 7.0) for 30 min. Sections were then rinsed in several changes of water over the next few hours to remove all endogenous sugars. Following rinsing, sections were incubated in the dark at room temperature in a reaction mixture comprised of equal volumes of 0.56 mg mL^{-1} phenazine methosulfate, 0.96 mg mL^{-1} nitro blue tetrazolium, 0.067 mL glucose oxidase mL^{-1}, and 20 mg mL^{-1} sucrose. All components of the mixture were prepared in a 0.38 M sodium phosphate (pH 6.0) solution. Sections incubated in the absence of sucrose served as a control for the reaction. Termination of the reaction after 30 min was accomplished by water rinsing and postfixing in 4% formalin for 15 min. Sections were rinsed several more times with water and stored in 15% ethanol at 4°C.

Enzyme Extraction

Tissue from mutant and normal kernels was homogenized in 5 mL of extraction buffer (gram fresh weight)$^{-1}$ with a mortar and pestle. Extraction buffer used in the isolation of soluble and particulate protein contained 50 mM Tris-maleate (pH 7) and 1 mM dithiothreitol (Doehlert and Felker, 1987). Homogenate suspensions were centrifuged at 14,000*g* for 10 min; the supernatant was removed and used as the soluble enzyme. The pellet was washed three times in extraction buffer succeeded by final resuspension in buffer containing 1 M NaCl. Bound enzyme was recovered in the supernatant subsequent to centrifugation at 14,000*g* for 10 min. Enzyme suspensions were dialyzed against extraction buffer overnight at 3°C.

Enzyme Assays

For invertase (EC 3.2.1.26) assays, 2 to 50 μL of dialyzed extracts were incubated for 15 min at 37°C in a reaction mixture containing 200 μL of 0.2 M sodium acetate, pH 4.8, and 50 μL of 0.04 M sucrose (Tsai et al., 1970). Samples incubated in the absence of sucrose served as controls. Enzyme reactions were terminated by the addition of copper-containing arsenomolybdate solution and quantitated by spectrophotometric absorbance at 660 nm. Specific activity of invertase is reported in micromoles per liter of glucose produced per milligram of soluble protein per minute.

Mixing experiments were done to monitor possible invertase inhibitors in homozygous *mn* kernels or in the upper parts of endosperm of the *Mn* genotype. Equal volumes of extracts corresponding to soluble or bound enzyme from tissue lacking such activity were mixed with those that showed abundant levels of enzyme activity, dialyzed overnight, and assayed similarly for enzyme activity. These values were comparatively analyzed against the mixtures with buffer.

ACKNOWLEDGMENTS

We are grateful to Oliver E. Nelson for stocks of *mn-1* reference and *mn-1* 7690 alleles and to Jack Shannon for stimulating discussions. This work was in part supported by USDA-CRGO No. 89-37261-4775 and USDA-NRICGP No. 91-37301-6343. This was a cooperative investigation of the U.S. Department of Agriculture–Agricultural Research Service and the Institute of Food and Agricultural Sciences, University of Florida (Agricultural Experiment Journal Series No. R-02059).

Received October 30, 1991; accepted January 6, 1992.

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