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DEVELOPMENT AND ASSIMILATE PARTITIONING IN
WILDTYPE AND MINIATURE PHENOTYPE MAIZE KERNELS

A Thesis in
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by
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ABSTRACT

Maize *miniure1* (*mn1*) is a seed specific maize mutant characterized by kernels deficient in invertase activity and with one fifth the mass of wildtype maize. Both self-pollinated wildtype and *mn1* plants and split-pollinated plants with *mn1* as the maternal parent were used to study development and assimilate partitioning in the maize kernel. Split-pollination simultaneously applies self and wildtype pollen to separate groups of silks on homozygous *mn1* plants to produce both wildtype and *mn1* phenotype kernels on a single ear. It facilitates comparative analysis of kernel development of two endosperm genotypes *mn1mn1mn1* and *Mn1mn1mn1* (designated *mn* and *wt* kernels, respectively) on a common *mn1* maternal plant. One dose of the *Mn1* gene is sufficient to restore kernels to full size (wildtype phenotype). The *wt* kernels not only resemble homozygous wildtype kernels in size but also in many physiological characteristics, such as endosperm cell number, starch granule number, endosperm cell nuclei endoreduplication pattern, presence of invertase activity at kernel base, and sucrose partitioning. Homozygous *mn1* kernels were significantly different from homozygous wildtype kernels in the pattern of assimilate compartmentation and partitioning in the growth period from 10 to 22 DAP (days after pollination). Similarly, differences in assimilate partitioning were found in *wt* and *mn* kernels of split-pollinated plants. Sucrose concentration did not differ between *mn* and *wt* kernels at 10 DAP, a period before strong sink development in maize kernels, but was higher in *mn* than *wt* kernels during rapid grain filling of *wt* kernels at 21 DAP. The differences of glucose and fructose specific activity between the two type kernels were significant at both 10 and 21 DAP. The difference in specific activity of sucrose between the two kernel types was significant only at 21 but not at 10 DAP. Significant differences in kernel tissue sugar composition and compartmentation were observed between homozygous wildtype and *mn1* tissues from 10 to 22 DAP. In pedicel tissue, homozygous *mn1* kernels had a higher percentage of sucrose and lower percentage of hexoses than wildtype kernels. That the difference in sugar percentage between the two genotypes became more dramatic in placental-chalazal
(P-C) tissue provides the evidence for invertase function. In wildtype kernels, because of the presence of invertase activity, a big pool of hexoses establishes in the apoplastic P-C tissue so that both sucrose concentration gradients between pedicel tissue and P-C tissue and hexoses concentration gradients between P-C tissue and distal endosperm are created and maintained. In endosperm tissue, homozygous wildtype kernels only had a lower sugar percentage than \textit{mn1} kernels. Tissue or sugar radioactivity in disintegrations per minute (DPM) relative to total tissue or carbohydrate DPM (relative DPM) was used in \(^{14}\text{CO}_2\) pulse-chase experiments with self-pollinated wildtype and \textit{mn1} plants to study carbohydrate transport kinetics. The difference in kernel sugar relative DPM was similar between the two genotypes for all tissues. The kinetics of sugar transport into kernels differed significantly between genotypes, but was similar among sugar species within a genotype. A low sucrose to hexose ratio in the phloem post-unloading zone is thought to facilitate sugar flux to sink tissue. The homozygous wildtype kernels had lower sucrose percentage to hexose percentage ratios than \textit{mn1} kernels in all kernel tissues, averaging 0.12 and 2.90, respectively, in P-C tissue. The amount of assimilate transported to \textit{mn} kernels was lower than that in \textit{wt} kernels when the leaves of split-pollinated plants were exposed to a pulse of \(^{14}\text{CO}_2\). The difference of assimilate partitioning is associated with the essential difference between the two kernel phenotypes, the existence of invertase in the pedicel and basal endosperm. In the split-pollinated plants, \textit{mn} kernels had fewer endosperm cells per kernel, fewer starch granules per endosperm and lower activities of starch synthesis related enzymes (AGPase and starch synthase) compared to \textit{wt} kernels. The \textit{mn} kernels had a higher level of ABA and lower level of IAA than \textit{wt} kernels through the 9 to 21 DAP growth period. There was no difference in cytokinin levels between the two kernel genotypes during most of the early developmental period, but \textit{mn} kernels had higher zeatin and ZR levels at 15 DAP and after 14 DAP, respectively, than \textit{wt} kernels. These results show that sucrose composition in kernel tissues, which is controlled by invertase at the kernel base, not only regulates assimilate transport to and partitioning in kernel tissue, but also interacts with other characteristics of kernel metabolism. Invertase at the kernel base plays a pivotal role in the growth and development of the maize kernel by affecting the final sugar mixture entering kernel sink.
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Chapter 1

LITERATURE REVIEW

1.1 General Introduction

Food deficiency is still a big problem in our world, especially in underdeveloped countries. Therefore, yield-improvement research in food crops, especially in major food crops such as corn, rice, wheat and soybeans, holds world-wide importance. Research has shown that crop yield is mainly determined by a crop's ability to produce high levels of photosynthate over a wide range of environmental conditions, and to efficiently transport and accumulate a high proportion of the photosynthate into economically important organs (Zamski, 1996). The amount of photosynthate flow from the various sources to a specific sink organ is influenced by photosynthesis and phloem loading in the source, translocation from source to sink along the axial path, and phloem unloading and utilization within the sink. Particularly in modern agriculture, the improvement of crop yield has been associated, not with an increase in total biomass production, but with a greater partitioning of the available carbon to the organs being harvested (Patrick and Offler, 1996). However, processes involved in regulating assimilate partitioning to harvest organs are difficult to understand because partition mechanisms apparently differ among crop species and even among the various sink tissues within a plant.

Maize (Zea mays L.) is the major, economically most important, domesticated plant in the United States and one of the major cereals in the world (Ritchie et al., 1993; Prioul, 1996). Maize is an important crop because it is not only used for human food and animal feed, but also for agro-industrial derivatives, such as starch, oil, proteins, and
cellulose. The harvested component which determines crop yield is usually grain, but harvest of whole shoot for silage is also economically important in northern areas of the United States. Appropriately, maize has received a considerable amount of attention from plant scientists through the years (Randolph, 1936; Kiesselbach and Walker, 1952; Shannon, 1968; Gengenbach, 1977; Shannon, 1982; Kowles and Phillips, 1988; Nelson and Pan, 1995; Neuffer et al., 1997). Considerable information has been obtained about maize anatomy, genetics and biochemistry. Assimilate allocation and partitioning, which highly influences maize yield, has been previously studied (Shannon 1968; 1972; Porter et al., 1985; Thomas et al, 1992; 1993), but the details of partitioning mechanisms and the regulatory factors involved are still lacking. Because of its importance in agricultural economy, maize kernel metabolism, especially the mechanism for storage product accumulation, deserves more attention. The maize kernel represents a well-defined system for analyzing post-phloem assimilate transport, sink metabolism and whole plant development. Maize has some special metabolic characteristics associated with its carbohydrate transport and accumulation in sink tissue that are different from other cereals, such as wheat (Doehlert and Lambert, 1991; Patrick and Offler, 1995; Thorpe and Minchin, 1996; Weber et al., 1998). Information on the mechanism(s) involved in phloem post-unloading during maize kernel development is incomplete. The complete function of invertase in the phloem post-unloading zone in relation to maize whole kernel development is still unclear.

The purpose of this thesis is to explore the mechanism of assimilate partitioning in the maize kernel by comparatively studying carbohydrate transport and accumulation in different genotypes of maize. The data obtained here will provide more insight about the regulatory factors affecting maize kernel growth.
1.2 Maize Endosperm

The study of maize kernel development is of great importance because of the prominence of maize in plant genetics research and its importance as a major agricultural food crop. From a genetic point of view, the maize kernel consists of three types of tissues: the maternal diploid tissue of the pericarp and pedicel, the filial triploid tissue of the endosperm and the filial diploid tissue of the embryo. Endosperm makes up 85-90% of the mature kernel dry weight and it is the major source of stored reserves within the mature seed. Therefore, endosperm tissue serves a critical role in overall kernel development and studying the development of the maize endosperm becomes especially important for understanding whole kernel growth.

1.2.1 Maize endosperm development

Maize endosperm is an unusual tissue. It is one of the two products of double fertilization. Maize pollen has two sperm cells. One sperm cell fuses with the egg cell, leading to the formation of the embryo. Endosperm comes from the fusion of the other sperm cell with central cell which has two polar nuclei. Unlike most plant cells that are diploid, the ploidy level of the endosperm is 3C (where C is the haploid nuclear DNA content at the G₀/G₁ phase of the cell cycle). Studies of the cytology and morphology of maize endosperm have indicated that endosperm development, starting with the formation of the triploid primary endosperm nucleus at the time of fertilization, consists of several sequential stages. They are mitosis, cell enlargement and differentiation, storage material accumulation, and desiccation and maturation (Lur and Setter, 1993a). Within several hours of fertilization (normally 2-4 hours), the central cell with its triploid nucleus undergoes rapid and synchronous divisions that continues for several days without cell wall formation (Kowles and Phillips, 1988; Kowles et al., 1990). Cell walls begin to develop between the nuclei at about 4 days following fertilization or about 5 days after pollination (DAP). By the fifth or sixth day after fertilization, the endosperm
is completely cellular and uninucleate (Kowles and Phillips, 1988; Olsen et al., 1998). The external form of the endosperm changes materially as it develops in the following days and there is a tremendous increase in size. For example, at 2 DAP, the approximate length of the endosperm is 0.3 mm, but at 8 DAP, it is 2 mm and at 12 DAP, it is 5 mm. Also there is a comparable increase in the diameter of the endosperm during the same period (Randolph 1936; Kowles and Phillips, 1988). The rapid change in overall endosperm size between 8 and 12 DAP is due to both an increase in cell number and, to some extent, nuclear and cell enlargement. Sucrose and hexoses accumulate in the ovule until the endosperm nuclei become surrounded by cell wall. Then the concentrations of these sugars decrease as they are used in cell wall formation and protein and starch synthesis in endosperm cells. Sucrose and other sugars transported through the phloem into endosperm cells provide for further endosperm growth and development. Kernel, endosperm and embryo dry mass reaches a maximum at around 50 DAP, depending on the genotypes and environment (Prioul, 1996).

The endosperm in its early stages is essentially undifferentiated and consists of thin-walled parenchyma cells of varying size and shape. Cell-division activity is general throughout the entire tissue, including both the marginal and the central cells. Gradually there is a tendency for cell division to be localized mainly in the peripheral zone and the growth of the inner region is primarily by increase in size rather than in number of cells (Randolph, 1936; Kowles and Phillips, 1988). Studies show that mitotic activity reaches a peak in the developing endosperm between 8 and 10 DAP (Kowles and Phillips, 1985). The mitotic index at this peak time is approximately 10%. Mitotic activity decreases sharply in endosperm cells 10-12 DAP. Mitoses are nearly absent in the centrally located endosperm cells when development reaches 12-14 DAP, whereas these activities persist for a longer time in the peripheral endosperm regions (Kowles and Phillips, 1985). Mitotic activity can be observed in the extreme peripheral tissue until about 20 DAP. The outmost layer of cells, the aleurone, cytologically behaves like a meristem providing additional cells by mitosis toward the interior region of the kernel (Olsen et al., 1998). Cells of the interior region and their nuclei, however, dramatically increase in size during
the early period of endosperm development (Kowles and Phillips, 1988; Kowles et al., 1990). Studies have shown that both nuclear divisions and cytokinesis eventually cease in the more central regions of the tissue by about 12 DAP (Kowles and Phillips, 1985; Myers et al., 1990; Jones et al., 1996).

1.2.2 Endoreduplication in maize endosperm cell

Even though mitotic activity stops in the centrally located maize endosperm cells at about 12 DAP, DNA synthesis continues in these cells (Kowles and Phillips, 1985). A dramatic surge in nuclear size and DNA content per nucleus normally occurs following the cessation of mitotic activity at about 10-12 DAP. Nuclear size and DNA content per nucleus sharply increase until peak levels are reached at about 14-18 DAP. DNA amplification during the 10-16 DAP period of development in the central region of the endosperm takes place at the phenomenal rate of approximately $2.75 \times 10^6$ base pairs per second per nucleus (Kowles and Phillips, 1985). This DNA amplification is the result of duplication of nuclear DNA in the absence of mitosis and cytokinesis (Kowles and Philips, 1988; Kowles et al., 1990; Grafi and Larkins, 1995). There is a positive correlation between nuclear volume and mean DNA content at each period of development. Analysis of DNA levels in the nuclei showed an exact doubling pattern indicating an endoreduplication process; it is an endonuclear chromosome duplication that occurs in the absence of mitosis, leading to the production of a chromosome with $2^n$ chromatids (Kowles and Phillips, 1985; 1988; Kowles et al., 1990; Grafi and Larkins, 1995).

Endoreduplication is common in tissues with high metabolic activity, such as the silk glands of dipterans and the developing endosperm of seeds (Grafi and Larkins, 1995). Endoreduplication occurs in a large proportion of the nuclei in developing maize endosperm and it appears to be an essential process in the development of this tissue (Grafi and Larkins, 1995). This process is not synchronous in maize endosperm tissue,
resulting in nuclei of various C levels. After endoreduplication, nuclei of maize endosperm cells can be classified as 3C, 6C 12C, 24C, 48C and 96C according to their DNA content. Endoreduplication coincides with the period of organelle proliferation and rapid expression of mRNAs for starch pathway enzymes and storage protein and is thought to increase the cell transcription capacity and enhance the events leading to cell differentiation (Lur and Setter, 1993b). The patterns of endoreduplication and extent of DNA amplification differ among some maize inbreds. Studies show that endoreduplication patterns in filial endosperm is more like the maternal parent than the paternal parent (Kowles, et al., 1997) and environmental stresses and plant hormones can influence maize endosperm nuclear endoreduplication and cell division (Kowles et al., 1992; Artlip et al., 1995; Engelen-Eigles et al., 1996).

Overall, the maize endosperm is extremely heterogeneous within a single kernel relative to cell size, nuclear DNA content, and, to some extent, morphological appearance (see section 1.5 on BETC). Since kernel development is fundamental to yield per se, understanding the plasticity of the nuclear genome and cell division related factors in endosperm during maize kernel development may lead to useful applications in genetic and breeding research.

1.3 Sink Strength and Factors Influence It

Sinks are plant tissues which do not synthesize all their own assimilate, but depend to some degree on importing it from the autotrophic tissues which are collectively called the source. The term sink strength was first introduced by Warren-Wilson in 1967 to describe the ability of sink tissue to attract and import assimilates. Although there have been long standing arguments about the definition of sink strength and its validity as a concept, it is still widely accepted in published articles and books (Wolswinkel, 1993; Zamski, 1996; Herbers and Sonnewald, 1998). For convenience, the concepts of sink and sink strength are still used in this thesis with their traditional meanings.
Essentially, all plant organs at some stage of plant development will act as sinks or receivers of assimilate (Ho, 1988). The major sink organ of maize varies along with plant developmental stage. Development of the maize plant can be divided into vegetative and reproductive stages. The vegetative stage is considered to extend from emergence to tasseling, even though reproductive structures are initiated by the plant long before the tassel emerges from the leaf whorl. The reproductive stage starts from silking and ends at physiological maturity (Ritchie et al., 1993). During most of the vegetative development period, the stem is the major sink. Toward the end of the vegetative stage, tassel and ear shoots become the major sinks. In the reproductive stage, developing kernels are the major sink. It has been shown that maize yield is correlated highly with individual kernel development and kernel numbers in each ear (Otegui and Bonhomme, 1998). One approach to increasing maize productivity is to increase the number and mass of harvestable kernels.

Although actual sink strength would be affected by the availability of assimilate supply and the proximity of the sink to the source, the most critical determinant is the intrinsic ability of the sink to receive or attract assimilate (Ho, 1988; Hebers and Sonnewald, 1998). This intrinsic ability of a sink is termed potential sink strength. It is genetically determined and can be fully expressed when the supply of assimilate is sufficient to meet the demand and the environmental conditions for the metabolic activity of the sink organ are optimal. Sink strength is generally considered to be the product of sink size and sink activity (Ho, 1988). Sink size is defined as a physical constraint and sink activity as a physiological constraint upon the sink organ's ability to import assimilate. The number of cells in the sink organ is considered to be a suitable measure of sink size. The sink activity refers to activities of all the enzymes associated with assimilate deposition inside sink organ. Sink activity is as important as sink size in determining sink strength. Many investigators have suggested that the overall physiological and metabolic processes occurring in the sink tissues are the main factors determining assimilate partitioning (Boyer 1996; Zamski, 1996).
1.3.1 Sink size in maize

Kernels are the reproductive sink of maize. Maize endosperm comprises approximately 83% of mature kernel volume and constitutes a majority of the kernel dry matter. Therefore, endosperm is the predominate sink for photosynthate and other assimilates and endosperm cell number can be used to measure maize kernel sink size. The cells of maize endosperm are primarily formed in the first 12 days following pollination with endosperm cell number reaching its maximum around 16 DAP (Kowles and Phillips, 1988). Endosperm cell number is a function of the rate and duration of cell division during the free nuclear phase of endosperm development (Kowles and Phillips, 1985; 1988). These parameters are genetically determined and are probably under the regulation of plant hormones. In non-stressed, optimal environments, plant hormones play an important role in regulating cell division and cell growth in plants. Roles of plant hormones, like auxin, cytokinins and ABA in maize endosperm development have drawn considerable attention in the past decade (Jones et al., 1990; Ober et al., 1991; Jensen and Bandurski, 1991; Jensen et al., 1992; Ober and Setter, 1992; Jensen and Bandurski, 1994; Cheikh and Jones, 1994; 1995; Artlip et al., 1995; Dietrich et al., 1995; Marion-Poll, 1997; Lejeune et al., 1998; Mambelli and Setter, 1998). It seems that there is no simple explanation of how plant hormones affect maize kernel development. Traditionally, the interaction of auxin and cytokinin in plant cell and tissue development has long been studied, but the regulation rule is complicate due to the variance of tissue (Coenen and Lomax, 1997). The ratio between auxin and cytokinin in a particular tissue tends to be more important than individual levels of auxin and cytokinin in determining the fate of the cell or of tissue development (Moore, 1989; Morris, 1996; Coenen and Lomax, 1997; Kende and Zeevaart, 1997).

The primary auxin in plants is Indole-3-Acetic Acid (IAA). The natural IAA exists in a variety of chemical states in plant tissue. IAA occurs not only in the free form but also conjugates to amino acids, peptides, or carbohydrates. Free IAA is considered to
be the form of auxin that is readily extractable and immediately usable in growth (Moore, 1989). The IAA conjugates are thought to be biologically inactive and appear to serve as IAA storage forms in seeds and hormonal homeostasis. In maize endosperm, there are both forms of IAA (Jensen and Bandurski, 1991; Jensen et al., 1992; Jensen and Bandurski, 1994). The level of free IAA tends to increase along with kernel development (Lur and Setter, 1993a).

Studies of cytokinin concentration in maize kernels have indicated that cytokinins are maximal at early growth stages, peaking between 4 and 12 DAP, a time period overlapping with endosperm cell division and the expression of plastid-specific proteins (Jones et al., 1990; Dietrich et al., 1995). High concentrations of cytokinins are found in the seeds of many species, where they are especially associated with tissues undergoing rapid cell proliferation (Chen, 1997 a,b; Kaminek et al., 1997; Prinsen et al., 1997). It has been observed that accumulation of cytokinins is correlated with the onset of cell division and concentration of cytokinins usually declines in tissues as rates of cell expansion decrease. It is generally thought that the free bases, such as isopentenyl adenine, zeatin and dihydro-zeatin, are the active forms of cytokinins. Some cytokinin derivatives also exist (Kende and Zeevaart, 1997). In maize kernels, ZR and zeatin are the most abundant and common forms of cytokinins (Jones et al., 1990; Dietrich et al., 1995). Whether all the cytokinins present in maize kernels are synthesized in situ or whether seeds also accumulate cytokinins transported to them in the xylem from the root system is unclear. Since cell number is an important contributor to potential sink strength, cytokinins may play a significant, if indirect, role in controlling potential sink strength of maize.

Another plant hormone, ABA, regulates diverse physiological processes throughout the plant life circle, such as modulating growth and differentiation of the embryo, enhancing synthesis of reserves in storage tissue and preventing precocious germination (Schussler et al., 1984; Belefant-Miller et al., 1994; Setter et al., 1998;
ABA mediated growth control is a fundamental response of plants to adverse environmental cues. Since cell ABA level normally increases in plants with exposure to different stresses such as drought, salinity or cold, ABA is considered as a signal for cells under stress. In most species, ABA content peaks during seed development at the time of seed maturation and the onset of dormancy. It has been reported that levels of ABA are lower during early stages of seed development than later stages, which is in contrast to that observed for cytokinins (Cheikh and Jones, 1994). In maize, ABA accumulates late in kernel development (Myers, 1990; Cheikh and Jones, 1994). Increasing ABA levels during the early growth period by exogenous application inhibits endosperm cell division and reduces the storage capacity of developing maize kernels (Myers, 1990). Gibberellic acid also accumulates during seed development in cereals, but does so later during the linear phase of growth and, therefore, presumably plays less of a role in the establishment of kernel sink capacity (Cheikh and Jones, 1994).

Apart from genetic determination, endosperm cell number can also be affected by external factors like environmental stress (Kowles and Phillips, 1988; Ober et al., 1991; Cheikh and Jones, 1994; Zinselmeier et al., 1999). The exact mechanism through which stress influences sink size is not well understood. However, changes in active hormone levels are likely to be one mechanism that produces quick responses. It appears plausible that the mechanism of sink size adjustment may involve changes in endogenous hormone levels since hormones coordinate the timing and rate of development among kernel constituent tissues and regulate overall rate of kernel growth. It has been found that when the environment for maize growth changes, levels of maize endosperm hormones and ratios among them are different from those in unaffected plants (Kuiper 1993; Lur and Setter, 1993b; Artlip et al., 1995; Ahmadi and Baker, 1999). Application of exogenous plant hormone to growing plants through stem infusion can alter sink development. Applying ABA to normally growing plants caused marked inhibition of photosynthesis and transpiration (Boyle et al., 1991) and applying synthetic cytokinin to heat-stress plants increased thermotolerance, in part by reducing kernel abortion at the tip and
middle positions on the maize ear (Cheikh and Jones, 1994). Plant hormones not only influence sink strength through cell division and differentiation, but they also directly affect assimilate partitioning into sinks. That the endogenous hormones of developing fruit may contribute to their capacity to import assimilates and to compete with other sinks is supported indirectly by a large number of observations. For example, dipping young, intact fruit into solutions of hormones (including GAs, cytokinins, and synthetic auxins) can substantially enhance their rate of assimilate import and dry weight gain (Morris, 1996).

The complexity of hormonal control in plants is displayed by interactions among different classes of plant hormones in the regulation of various developmental and physiological processes. It is becoming increasingly evident that these interactions also involve alterations of one hormone level by another. The amount of plant hormone in a plant organ is dynamic, determined by the combined rates of its biosynthesis, breakdown, import and export. In addition, the concentration of each hormone is under the control of poorly understood metabolic regulation responding to its own level in plant cells (Kaminek, et al., 1997; Bennett et al., 1998). Cytokinins and auxins interact in the control of many developmental processes in plants such as cell division and differentiation, organ formation in cultured tissues, apical dominance and leaf senescence. Recent studies, such as with the ipt gene in transgenic plants and exogenous application of auxins to plant tissue, have provided more evidence of cytokinin and auxin interactions (Chen, 1997a; Kaminek, et al., 1997). It has been proposed that down regulation of cytokinin levels by auxin may occur through the inhibition of cytokinin biosynthesis, or the promotion of cytokinin metabolic inactivation by N-glucosylation or through degradation by cytokinin oxidase (Kaminek, et al., 1997). On the other hand, the accumulation of cytokinins have little effect on auxin levels except to increase the cytokinin/auxin ratio which induces in competent plant cells certain physiological and/or structural processes (Chen, 1997a; Kaminek, et al., 1997). In addition, plant hormones also influence each other by affecting gene expression. Physiological, biochemical, and
genetic evidence indicate that cytokinins play an important role in regulating plant gene expression by selectively increasing or decreasing the abundance of specific proteins or mRNAs (Chen, 1997b). Experimental evidence demonstrates that cytokinins regulate gene expression at the level of transcription and/or post-transcription (Chen, 1997b). In many instances, cytokinins interact with other hormones or environmental factors in controlling developmentally regulated genes, including cell cycle genes. Cytokinin-binding proteins have been isolated from plant cells, but their roles in signal transduction are still unknown. Regulation of gene expression by plant hormones during development includes a cascade of events with altering sequences of regulatory gene expression (Chen, 1997b). Therefore, it is not only the amount of a specific hormone, but also the balance between hormones that determines the final physiological response of the organ.

1.3.2 Sink activity

Sink activity refers to internal physiological processes that affect the sink organ’s ability to import assimilate and it is expressed in activities of enzymes that relate to sink size establishment. Sink formation is comprised of three important physiological processes: firstly, the unloading of photoassimilates from the phloem, post-phloem transport, and retrieval by sink cells; secondly, assimilate utilization, mainly by respiration, and thirdly, storage of imported carbohydrates (Herbers and Sonnewald, 1998). Sink activity is as important as sink size in determining sink strength. Import rate of assimilates is not entirely determined by the size of the sink, but it is regulated also by the metabolic activity of the sink organ. There are many metabolic processes going on in the maize kernel with the end result being the storage of reserves, like starch, protein and oil.

Starch synthesis in storage tissues appears to be a very efficient process (Shannon, 1974; Morell et al, 1995; Kleczkowski, 1996; Preiss and Sivak, 1996; Wang et al, 1998). Young maize endosperm cells are highly vacuolated. At 10 to 12 DAP, small starch
granules are initiated fairly synchronously in the cytoplasm surrounding the nuclei of endosperm cells. These starch granules are produced in specialized organelles called amyloplasts. Starch is initially formed in the central crown region of the endosperm and starch synthesis proceeds centrifugally and toward the kernel base (Boyer and Shannon, 1986). In mature maize kernels, the large starch granules reach about 20 µm in diameter in about 20 days after their initiation (Shannon, 1974). Since starch is the main component of a maize kernel, about 88% of endosperm, sink strength during grain filling is mainly controlled by the efficiency of the starch-synthesizing enzymes. Activities of enzymes involved in starch synthesis are used as the parameters indicating sink activity in maize (Morell et al., 1995; Kcleczkowski, 1996; Preiss and Sivak, 1996; Wang et al., 1998). Many biochemical and molecular studies have demonstrated the rate-limiting enzymes and genetic regulatory steps involved in maize starch synthesis, such as Adenosine Diphosphate-glucose Pyrophosphorylase (AGPase), soluble and bound starch synthases, starch branching enzyme and starch debranching enzyme (Echeverria et al., 1988; Doehlert and Kuo, 1990; Felker et al., 1990; Doehlert and Chourey, 1991; Guy et al., 1992; Cao et al., 1995; Chourey et al., 1995; Miller and Chourey, 1995; Nelson and Pan, 1995; Shannon et al., 1996; Dejardin et al., 1997). Recent studies have shown that AGPase is the key regulatory enzyme of starch biosynthesis in green and nongreen tissue of higher plants (Ziegler, 1990; Prioul et al., 1994; Denyer et al., 1996; Singletary et al., 1997; Greene and Hannah, 1998). AGPase (EC 2.7.7.27) catalyzes a reversible synthesis of ADP-glucose and PPi from ATP and glucose-1-phosphate (G1P). The ADP-glucose will be further used by starch synthase to synthesize amylose. After the function of starch branching enzyme and starch debranching enzyme, amyllopectin are formed and packed in amyloplast as granules. AGPase is the rate-limiting enzyme (Chen et al., 1998; Greene and Hannah, 1998). Its activity highly influences the amount of starch in kernels and this enzyme is finely regulated by substrates and various other signals that regulate endosperm cell growth and development.

Maize kernel is a storage sink and its growth includes two stages: increase in cell number and accumulation of storage compounds, mainly starch. Sink storage capacity is
established during the early stage of grain development and depends on the processes of cell division, organelle proliferation and cell enlargement, which collectively determine the metabolic activity and final volume of kernels. In maize, the final grain dry weight is essentially determined by the number of cells in the endosperm and the number of starch granules in each endosperm cell (Jones et al., 1996). Indeed, cell division activity is crucial in attracting assimilate to sink organs in the early stages of development. Import rate of assimilates into maize kernels is determined by both sink size and metabolic activity of the sink organ during development.

1.4 Phloem Unloading and Post-unloading

The source of assimilates for maize plant growth during most developmental stages is the leaves. Assimilates have to go through phloem loading, phloem unloading and post-unloading (also called post-sieve element transport) in order to reach distant sinks from the source (Oparka, 1990; Patrick and Offler, 1995). Carbon compounds derived from photosynthesis move out of chloroplasts and are used to synthesize sucrose in the cytosol of maize bundle sheath cells. Sucrose moves through the apoplasm to the vicinity of the sieve elements in the smallest veins of the leaf. Subsequently, sucrose is actively taken up into the sieve element/companion cell complex, a process named phloem loading (Delrot, 1989; Turgeon, 1996). Sucrose has been shown to be the major form of sugar transported in maize phloem (Shannon, 1972; Prioul, 1996). As sucrose is actively accumulated in the phloem, osmotic pressure within the sieve elements increases, causing water to enter the phloem and this apparently generates the driving force for the mass flow of assimilates from the source leaf (Giaquinta, 1980). A high hydrostatic pressure, which can reach 30 atmospheres, develops within the sieve element and is believed to power the mass flow of the loaded molecules and water to regions of phloem unloading where hydrostatic pressure is lower (Oparka et al., 1994). Once sucrose is loaded into phloem, it is translocated through the vascular system to different sink tissues for allocation and partitioning.
1.4.1 Phloem unloading

Grain filling is a critical determinant of yield in many crops. The process depends on the rate and efficiency of two different mechanisms: phloem unloading, and the uptake and utilization of unloaded assimilates for seed growth and storage product formation (Wardlaw, 1990). Previous studies have demonstrated that maize kernel development is dependent on a supply of phloem transported sucrose (Felker and Shannon, 1980; Porter et al., 1985). Nutrients are unloaded from the phloem sieve elements located in the maternal pedicel tissue at the base of the kernel. Vascular elements split and anastomose throughout the pedicel. At their termination, the sieve elements become indistinguishable from parenchyma cells which are interconnected by plasmodesmata (Felker and Shannon, 1980). The parenchyma of the pedicel provides a symplastic route for sucrose distribution throughout the pedicel parenchyma. Assimilate is then passively unloaded into the apoplasm of the pedicel parenchyma (Porter et al., 1986; Shannon et al., 1986; Porter et al., 1987). This movement of sucrose out of phloem terminal sieve elements into the tissue symplasm and then into the tissue apoplasm of the pedicel is called phloem unloading.

1.4.2 Phloem post-unloading

After phloem unloading, sucrose passes through additional maternal tissue before reaching the filial tissue of the kernel. The maternal tissue directly between the pedicel parenchyma and the endosperm of the filial tissue is called the placento-chalazal (P-C for short) tissue. P-C tissue consists of 10 or more layers of rectangular cells. The cytoplasm of the P-C cells begin to deteriorate rapidly around 7 DAP (Shannon, 1978; Shannon et al., 1986; Miller and Chourey, 1992). Within a few days these cells appear dead. Plasmodesmatal channels remain in P-C cell walls indicating that assimilates may move symplastically and/or apoplastically before cell death and apoplastically after cell death (Shannon, 1978). Because the cytoplasm of the pedicel parenchyma cells is
interconnected via plasmodesmata (Shannon et al., 1986), the unloaded sucrose can easily move from pedicel parenchyma to P-C tissue. Uptake of assimilates by the endosperm from the P-C apoplastic space is facilitated by the conversion of the cells at the base of the endosperm into transfer cells, which are anatomically and functionally distinct from the other endosperm cell types (Shannon et al., 1986; Davis et al., 1990; Hueros et al., 1995). Since the sucrose degrading enzyme, invertase, widely exists in the distal pedicel (near the endosperm) and basal endosperm region, sucrose may be hydrolyzed into glucose and fructose anytime after phloem unloading. Consequently, it is a mixture of sucrose and its monomer derivatives that move into the endosperm from P-C tissue. Post-unloading sugar transport from pedicel parenchyma to P-C to the basal endosperm cells appears to occur passively (Porter et al., 1985; 1986; Griffth et al., 1987; Oparka, 1990). The distal part of pedicel parenchyma, the P-C tissue and the basal endosperm transfer cells (BETC) are named the post-unloading zone.

Sucrose, the main form of sugar in maize remains intact as a disaccharide all the way from phloem loading at the source to phloem unloading at the post-unloading zone. It has been suggested that sucrose inversion in the post-unloading zone may regulate the rate of phloem unloading by maintaining a steep sucrose concentration gradient between the source and the post-unloading zone. In addition, sucrose hydrolysis produces glucose and fructose and may increase their concentration gradients between the post-unloading zone and endosperm tissue (Shannon, 1972; Miller and Chourey, 1992; Shannon et al., 1993). These sugar concentration gradients between the post-unloading zone and endosperm tissues have been proposed to produce the driving force for continuous assimilate transport to the endosperm sink. Sucrose resynthesis in the endosperm may maintain a favorable gradient for passive transfer of hexose into the endosperm. However, the rate of starch accumulation in maize endosperm is normally limited not only by the supply of assimilate but also by the activity of the starch-synthesizing enzymes in the endosperm cells (Ho, 1988). The passive uptake of sugar into the endosperm and its regulation by the activity of endosperm starch synthesis were confirmed by Griffith et al. (1987a) in short-term transport studies with excised kernels.
Assimilate transport and partitioning in sink tissue are also affected by environmental factors (Geiger et al., 1996). It has long been recognized that the import rate of a sink can be reduced by local application of low temperature, anoxia, or a metabolic inhibitor, resulting in reduced growth (Ho, 1986). Many investigators have suggested that the overall physiological and metabolic processes taking place in the sink tissues are the main factors determining assimilate partitioning to plant organs (Zamski, 1996).

1.5 Basal Endosperm Transfer Cells (BETC)

As all assimilates for development of endosperm, embryo and other kernel tissues proceed through post-unloading, understanding the structures involved in post-unloading is important. A group of specialized endosperm cells develop adjacent to the P-C tissue of the pedicel. These cells acquire the characteristics of transfer cells, including extensive fingerlike cell wall extensions that protrude into the cell lumina. This characteristic increases the surface area of the associated plasmalemma up to 20-fold (Davis et al., 1990). These cells are referred as basal endosperm transfer cells (BETC) (Kiesselbach and Walker, 1952; Felker and Shannon, 1980; Davis et al., 1990). The overall shape of the BETC zone is concave with 50 or more cells width and 3-6 cells depth. The BETC zone is bounded one side by starchy endosperm cells, on the other side by crushed remnants of 10 or more layers of maternal cells (most of them are P-C cells) and at the edges by aleurone cells. All the cells associated with this zone have primary cell wall (Davis et al., 1990). Even though these BETCs are very different in structure from the aleurone cells that surround the endosperm, they are contiguous with the surrounding cells (Shannon, 1982). An internal cuticle surrounds the entire caryopsis and extends into the area below the transfer cell zone, but is not continuous through the P-C region (Shannon, 1982; Davis et al., 1990). Since there are no plasmodesmatal connections between the P-C tissue and the BETC, it appears that all metabolites that enter the grain must enter the BETC from the apoplastic space of the P-C tissue. Once the sugars pass through the cell wall of BETC and get into its cytoplasm, they move
symplastically throughout the endosperm. Numerous plasmodesmata have been shown to exist between endosperm cells (Felker and Shannon, 1980; Davis et al., 1990).

It is of considerable interest to study maize BETC, especially its function during post-unloading, because of the special location and characteristics of these cells. Transfer cells have been shown to be important in many different plants. Transfer cells are known to be actively involved in intensive, short distance, intercellular transport of assimilates in some grain caryopases (Davis et al., 1990). It is likely that the function of maize BETC is to absorb metabolites from the maternal tissue and get them into the symplastic pathway of the endosperm cells. Results from anatomical studies support this hypothesis. The initiation of basal endosperm differentiation in maize was observed around 6 DAP (Kiesselbach and Walker, 1952; Shannon, 1978; Felker and Shannon, 1980). BETC wall ingrowths develop rapidly during the next few days and become very extensive by 20 DAP (Shannon, 1986). The timing of wall ingrowth formation fits the initiation of the fast growth period of maize kernel development. It is known that the first 8 to 12 DAP is a critical period during kernel development in maize because several developmental events during this period are important determinants of the fate of subsequent kernel growth and development (Jones et al., 1996).

Davis et al. (1990) reported a detailed electron microscopy study of maize BETC at 23 DAP. They reported that BETC had numerous cell-wall extensions in the form of anastomosing lamellae and the cytoplasm of BETC, rich in mitochondria, filled the interstices of cell-wall extension. They also found many vesiculate areas along the plasma membrane. All these cytological characters support high activity in metabolism of these cells. The authors emphasized that BETC are presumably the first nonmaternal cells that metabolites must contact before entering the endosperm (Davis et al., 1990).

Recently a gene expressed in the basal endosperm transfer cells of maize, \textit{BET1}, was identified (Hueros et al., 1995). The \textit{BET1} was isolated from a cDNA bank prepared from mRNA of 10-DAP maize endosperm. The expression of \textit{BET1} was shown by
Northern blot to commence at 9 DAP. Three species of the antibodies raised against the BET1 protein appeared to be basal endosperm cell specific. This result and the reactivity of exhaustively extracted cell wall material with the BET1 antibody suggest that the structural specialization of the basal endosperm transfer cells is genetically controlled even though the exact function of BET1 in specialization of BETC is still under investigation (Hueros et al., 1995).

Because of the strategic location of BETC, its functional role of facilitating solute influx to endosperm is widely accepted, despite the fact that BETC are crushed by the enlarging embryo at the end of kernel development, around 32 to 40 DAP. Shannon (1986) reported that the death of the placento-chalazal cells adjacent to the BETC begins around 7 DAP, and that the death coincides with the initiation of wall ingrowth of the BETC. Thus, during the period of active kernel growth and starch deposition, the BETC with the layer of subtended dead cells effectively increases the free space volume in the site of assimilate post-unloading. This free space is thought to facilitate the assimilate absorption from the pedicel by the endosperm. Obviously more physiological study of BETC is needed before its function in post-unloading can be fully understood.

1.6 Invertase

Invertase (β-fructofuranoside fructohydrolase, EC 3.2.1.26), a sucrose degrading enzyme, is widely distributed in maize tissues, including roots, leaves, stems, silks, kernels and some other tissues (Sturm, 1999). There are at least two forms, soluble and insoluble invertase, which are common in most organisms. The soluble form is predominantly localized to vacuoles and cytoplasm, and its optimal pH for activity is 5 to 5.5 (for the acid form) and 7 to 8 (for the neutral form). The insoluble form, in all cases tested, is bound to the cell wall (hence also called a cell-wall-bound form), and its optimal pH is 4.5 (Ho et al., 1991). Invertase hydrolyzes sucrose into fructose and
glucose, and the reaction is irreversible. This is different from the reversible transglycosylation reaction by sucrose synthase (Quick and Schaffer, 1996).

Invertase is believed to play an important role in sucrose mobilization between source and sink organs of plants even though physiological roles of invertase in various tissues are not well understood (Eschrich, 1989; Sung et al., 1994; Taliercio et al., 1995; Weber et al., 1995; Sturm and Tang, 1999). Because insoluble invertase is widely found in the cell wall of growing tissue and would be activated at about pH 4.5 and inactivated above pH 6.5, the activity of the cell-wall-bound invertase has been suggested to be a factor regulating the rate of assimilate unloading (Eschrich, 1980; Shanker et al., 1995). It has been proposed that sucrose is unloaded from phloem sieve elements into the cell wall free space intact and that it will remain intact at neutral pH. But this sucrose can re-enter the phloem. The free space insoluble acid invertase could act as a type of "reflux valve" by splitting sucrose into its hexose moieties and preventing sucrose reloading into the phloem (Eschrich, 1989). Recent molecular research has provided more evidence supporting the concept that insoluble invertase is important for sucrose transport (Stitt and Sonnewald, 1995; Caimi et al., 1996; Tang et al., 1999). In both tomato and tobacco transgenic plants, expression of a chimeric invertase gene targeted into the cell wall led to serious interruption in sucrose loading into the sieve element/companion cell complexes and export, leading to inhibition of growth (Schaewen et al., 1990; Dickinson et al., 1991). Consistent with this result is the genetic data from cultivated and wild relatives of tomato which shows that the accumulation of sucrose or hexoses in ripening fruits is entirely dependent on a monogenic trait related to acid invertase expression in this tissue (Chetelat et al., 1993; Klann et al., 1993). Results obtained with the expression of yeast invertase in potato tubers indicate that tuber apoplastic space is involved in sucrose utilization, so sucrose hydrolysis might be involved in the determination of sink strength (Frommer and Sonnewald, 1995). Koch and Noltz (1995) concluded that a good portion of translocated sugars still enter some organs to participate in cellular metabolism through the enzymatic reaction of insoluble invertase working in extracellular space even though the extent and essentiality of hydrolysis to import has been debated. Meanwhile,
soluble invertase has been found in the vacuole of sucrose-storing tissues during mobilization of sucrose (Boller and Wiemken, 1986). The role of soluble invertase in assimilating source tissues is less clear, although it may mobilize sucrose temporarily stored in the vacuoles (Obenland et al., 1993; Carlson and Chourey, 1999). In addition, soluble invertase activity was suggested to be especially important during cell expansion, such as occurs during leaf enlargement, early fruit/seed development, and in rapidly elongating tissues (Xu et al., 1995). Genes, named *Ivr1* and *Inv2, Incw1* and *Incw2*, have been identified for soluble and insoluble maize invertase, respectively (Shanker et al., 1995; Xu et al., 1995; Cheng et al., 1996; Xu et al., 1996; Carlson and Chourey, 1999). Their functions in kernel development are still under investigation.

Maize kernels have both forms of invertase. Histochemical staining of invertase activity in kernels from 7 to 42 DAP showed a constant presence of invertase in all the basal endosperm tissues, such as pedicel parenchyma and BETC and in the scutellum at the base of the endosperm (Doehlert and Felker, 1987; Miller and Chourey, 1992). But no activity could be detected in the embryo or middle and crown part of the endosperm (Doehlert and Felker, 1987; Miller and Chourey, 1992; Prioul, 1996; Zhou et al., 1997). The role of invertase in maize kernels is of significant importance as it is entirely localized in the basal endosperm area and is developmentally the earliest enzyme with the highest specific activity levels in the endosperm (Chourey et al., 1995). It has been proposed that invertase activity maintains the driving force for assimilate movement into developing kernels by maintaining a sucrose-gradient between the terminal phloem cells and the pedicel apoplasm and by generating a hexose concentration gradient between the pedicel apoplasm and the starchy endosperm cells (Shannon et al., 1993). Shannon (1972) concluded from a 14CO2 labeling experiment that sucrose is hydrolyzed into glucose and fructose by invertase in the pedicel region prior to absorption into endosperm by basal endosperm transfer cells. Subsequently, many physiological studies have provided evidence for the existence of sucrose inversion and the involvement of invertase function in maize kernel development (Felker and Shannon, 1980; Porter et al., 1985; 1986; 1987; Doehlert and Felker, 1987; Miller and Chourey, 1992; Shannon et al., 1993;
Cheng et al., 1996). On the other hand, Schmalstig and Hitz (1987) reported that intact maize kernels can absorb the sucrose analog, 1'fluorosucrose, into endosperm without inversion by invertase. An argument that sucrose hydrolysis is not a prerequisite for the unloading and subsequent massive import of sugar into the maize endosperm sink cells arose from this finding. So far, the need for invertase in maize kernel post-unloading zone has not been clarified, even though several investigations have studied it (Porter et al., 1986; Doehlert and Felker, 1987; Miller and Chourey, 1992; Cheng et al., 1996; Xu et al., 1996; Carlson and Chourey, 1999; Cheng and Chourey, 1999).

1.7 The miniature1 Mutant

The miniature1 (mn1) maize mutant was first described as a seed specific mutation by Lowe and Nelson (1946). The vegetative phenotype of the mn1 maize plant is the same as that of the wildtype. But mn1 kernels are much smaller in size and have a papery, top-dented pericarp. Kernels of the mn1 genotype cease growth around 12-13 DAP and only weigh about one fifth of the wildtype kernels at maturity (Lowe and Nelson, 1946). More recently, mn1 maize was characterized as being defective in cell-wall bound invertase in basal endosperm and pedicel tissue (Miller and Chourey, 1992). This group later showed that mn1 is a null mutation of the wildtype gene, Mn1, which encodes a cell-wall bound invertase isozyme (Cheng et al., 1996). Significantly, this single gene mutation not only eliminates invertase activity in the filial basal endosperm tissue, but also in the maternal P-C tissue of the pedicel. Genetic analyses suggested that the loss of enzyme activity in endosperm tissue may be the causal basis of its loss in the pedicel (Cheng et al., 1996). The mn1 seed mutant is unusual because a single gene mutation affects both the filial and the maternal generations of the plant. It also is the first invertase deficient mutant identified in higher plants.

In addition to being defective in invertase, mn1 maize kernels also develop an early degeneration and separation of maternal cells from BETC (Miller and Chourey,
The onset of tissue separation (called a pedicel gap) occurs around 9 DAP. Before 9 DAP, there are no detectable anatomical differences between \textit{mn1} and wildtype kernels. The structural aberrations appear to be restricted to maternally derived cells of the placento-chalazal region. No detectable structural aberrations were found in BETC or pedicel parenchyma cells (Miller and Chourey, 1992). The formation of the pedicel gap in the \textit{mn1} mutant is not simply the consequence of a passive withdrawal of pedicel tissue away from endosperm tissue, but it is associated with significant cell rupture and degeneration (Miller and Chourey, 1992). Close examination of cells affiliated with PC tissue revealed cell wall degeneration and remnants of broken cells in the region normally marked by a physical contact between the pedicel and the endosperm (Miller and Chourey, 1992). In addition, the developing \textit{mn1} endosperm begins to separate from distal pericarp around 8 to 10 DAP, a process that continues to seed maturation. The physiological causes of these anatomical lesions are still unknown (Cheng et al., 1996).

Another maize mutant, \textit{mn1-89}, generated by ethyl methanesulfonate (EMS) mutagenesis from \textit{mn1} mutant kernels, provides additional insight of the roles of invertase in kernel development (Cheng et al., 1996). The homozygous \textit{mn1-89} mutant kernels only have about 6\% of the total invertase activity of wildtype kernels. The phenotype of the homozygous \textit{mn1-89} mutant kernel is similar to the wildtype except for a slight loss of seed weight and size. The phenotype of \textit{mn1-89} maize plants is the same as the wildtype; the height of the plant and the number of leaves when plant reaches maturity are similar between the two plant types and there is no pedicel gap formation in \textit{mn1-89} mutant kernels (Cheng et al., 1996). The study of these available genotypes has shown the relationship between invertase levels and the normality of maize kernel development (Cheng et al., 1996). Results from reciprocal hybrids between \textit{Mn} and \textit{mn1}, \textit{Mn} and \textit{mn1-89}, and \textit{mn1} and \textit{mn1-89} imply that a substantial amount of invertase activity found in wildtype plants is dispensable (\textit{Table 1–1}). There appears to be a threshold value of 4\% of the wildtype invertase activity below which invertase played a rate-limiting role in the normal development of the kernel, including the developmental stability of the maternal cells (Cheng et al., 1996).
The \textit{mn1} maize mutant can be used as a tool to study assimilate partitioning during maize kernel growth and development because it has such unique characters in enzymology and anatomy. It should be possible to investigate the factors that regulate and control assimilate partitioning during maize kernel growth and development by a comparative study of wildtype and \textit{mn1} kernels. Physiological studies show that at 12 DAP, there is no difference in the total sugar composition of the wildtype vs. \textit{mn1} mutant pedicel, but endosperm tissue of the \textit{mn1} kernels contained only 42\% as much soluble sugar (sucrose, glucose and fructose) as wildtype kernels (Shannon et al., 1993). In both tissues from the wildtype kernels, sucrose accounted for about 62\% of the total soluble sugars, while in the \textit{mn1} kernels the sucrose percentage was about 85\%. In both genotypes the balance in monomer sugars was relatively evenly distributed between glucose and fructose. These data clearly show that sucrose inversion is indeed reduced and assimilate partition into the endosperm is less in \textit{mn1} compared to wildtype kernels (Shannon et al., 1993). It has been suggested that a physiological gradient of photosynthate between pedicel and endosperm is mediated by invertase (Miller and

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
Endosperm genotype & Endosperm total invertase activity (µmol reducing sugar/mg protein/min) & Pedicel gap \\
\hline
\textit{Mn1Mn1Mn1} & 100 & No \\
\textit{Mn1Mn1mn1} & 78.6 & No \\
\textit{Mn1Mn1mn1-89} & 73.7 & No \\
\textit{Mn1mn1-89mn1-89} & 22.2 & No \\
\textit{Mn1mn1mn1} & 21.0 & No \\
\textit{mn1-89mn1-89mn1-89} & 6.5 & No \\
\textit{mn1-89mn1-89mn1} & 4.3 & No \\
\textit{mn1-89mn1mn1} & 2.4 & Yes \\
\textit{mn1mn1mn1} & 1.8 & Yes \\
\hline
\end{tabular}
\caption{Comparative levels of total invertase activity at 12 DAP from kernel extracts from homozygous wildtype (\textit{Mn1}), homozygous \textit{mn1}, \textit{mn1-89} and heterozygous genotypes obtained by reciprocal crosses (reconstructed from data in Cheng et al., 1996).}
\end{table}
Chourey, 1992; Shannon et al., 1993). These authors believe that the maintenance of an assimilate gradient is required to support structural stability of pedicel maternal cells as well as to maintain normal development of endosperm tissue and the maize kernel (Miller and Chourey, 1992; Shannon et al., 1993; Cheng et al., 1996). As yet, no physiological study has been done on kernel sink strength and assimilate partitioning with these mutants.

1.8 Sugar Sensing

Photosynthesis distinguishes plants from all other organisms. Sugar production through photosynthesis is the most fundamental activity in plant life. The process of sugar production, transport, consumption, and storage are dynamic and tightly linked to cellular physiology, organ identity, environmental conditions, and developmental stage. In plants, sugar has been viewed conventionally as a resource for respiration and metabolic intermediates, as well as a source of structural or storage components. Sugars not only function as substrates for growth, but it is now thought that they play a substantial role as a signal compound involved in the regulation of certain cellular processes, such as enzyme activity, metabolism, development, and gene expression through various sugar-sensing systems (Koch, 1996). The former concept is easily understood, but the latter one is more complicated and obscure. Sugar sensing is one of the most novel topics in current biology. Sugar sensing and sugar-regulated gene expression in higher plants have become prominent topics in recently years (Koch, 1996; Jang and Sheen, 1997; Smeekens and Rook, 1997; Chiou and Bush, 1998; Sheen et al., 1999). Recent compelling evidence supports the concept that sensing and signaling can be performed even at a millimolar range of signaling molecules using sugar binding enzymes, proteins, or transporters (Sheen, et al., 1999). A plant’s ability to monitor and respond to sugar levels could serve as a control mechanism to integrate external environmental conditions including light, other nutrients, and abiotic and biotic stresses, with intrinsic developmental programs directed by multiple plant hormones.
Sugar has been shown to be involved in maintaining normal development of tissues and organs, regulating enzyme activities, and gene transcription and expression (Koch, 1996; Jang and Sheen, 1997; Smeekens and Rook, 1997; Chiou and Bush, 1998; Sheen et al., 1999). Sugar sensing and signaling in photosynthetic plants involves a complicated network of processes. In principle, a large number of different sugars and intermediates in sugar metabolism can effect expression of sugar-regulated genes. To date, however, such a signaling function has been proposed for only a few sugars. Hexoses are sugars of clear importance in this respect and a signaling function has been proposed especially for glucose. Hexoses can modulate gene expression by entry into the plant cell or by entry into intermediary metabolism through the action of hexokinases (Smeekens, 1998). Sucrose is the most frequently used sugar in studies of plant sugar responses in gene regulation and development because sucrose is the main form of sugar transport through the plant phloem system. Beside its role to fuel growth and development of plants, sucrose is believed to act directly or indirectly as a mediator between source and sink tissues, regulating photosynthesis according to sink demand. Sucrose may regulate genes encoding storage proteins, enzymes involved in starch metabolism, and activate some defense related genes. In some cases, the effects of sucrose can be completely substituted by those of hexose, such as glucose and fructose (Sheen, et al., 1999). These results indicate that in many cases sucrose is not the direct signaling molecule. However, sucrose could have a signaling mission distinct from that of hexoses, and could be perceived by different types of sensors/receptors in a signaling pathway. For example, sucrose, and not hexoses, has been shown to regulate the transcript level of a sucrose symporter in sugar beets (Chiou and Bush, 1998).

Metabolites as signals have been mainly neglected until recently when sugars were shown to act as important components in signal transduction networks (Koch, 1996; Jang and Sheen, 1997). A close interaction between sugar signaling and developmental processes has been observed (Weber, et al., 1996; Jang and Sheen, 1997; Smeekens and Rook, 1997; Chiou and Bush, 1998; Wobus and Weber, 1999). In a study with *Vicia*
faba developing seeds, it was suggested that hexoses signal meristematic activity (cell division) in the developing cytoledons, whereas sucrose induces a switch towards the non-proliferative storage phase of seed development (Weber, et al., 1996). Upon fertilization, an invertase associated with the seed coat generates hexoses from incoming sucrose. The duration of expression of this invertase activity is a determinant for the number of embryo cells and eventually it determines storage capacity and seed size (Weber, et al., 1996). If metabolites are able to act as signals in developmental processes, the regulated import of photoassimilates into the growing seed will influence seed development in addition to providing nutrients.

Metabolites have been shown to regulate diverse gene activities in plants. The control of enzymatic activity by sugars and sugar metabolites has been investigated in detail and these studies have yielded insights into the regulation of metabolic pathways (Koch, 1996; Jang and Sheen, 1997; Smeekens and Rook, 1997; Chiou and Bush, 1998; Smeeken, 1998; Sheen et al., 1999). It has also been shown that in these pathways several different enzymes usually share in flux control instead of it being regulated by a single rate-limiting step. The expression of a large number of genes is altered by changes in sugar levels. These genes encode proteins that function in carbohydrate metabolism and, equally important, in many other metabolic pathways and developmental programs. Sugar regulation of gene expression can be mediated at the transcriptional and post-transcriptional levels (Sheen et al., 1999). Plant sugar sensing and signaling pathways are mediated by multiple sensors and linked to various aspects of plant growth and development. The underlying regulatory mechanisms are probably too complex to be completely elucidated by molecular and biochemical approaches. The power of genetics in uncovering surprising components and cross-talk among pathways will provide new and complementary means for dissecting the sugar signal transduction pathways by using a wide range of sugar mutants, like those found in Arabidopsis thaliana (Smeeken and Rook, 1997).
The picture which emerges is that a sugar-responsive regulatory web in which endogenous developmental programs and external stimuli are integrated and result in a coordinated metabolic response. It is, therefore, of great interest to understand the way in which sugars are sensed and how this sensing activates signal transduction pathways leading to altered gene expression. These sugar-sensing and signal transduction systems will interact closely with pathways responsive to other stimuli like phytohormones and light. In the present study, I investigated the difference between wildtype and \mn1 phenotype kernels in endosperm cell number, starch granule number in endosperm cells, endosperm cell nuclear endoreduplication, activities of enzymes associated with endosperm starch synthesis, plant hormones in endosperm cells, and distribution of radioactive assimilates after exposing leaves to a pulse of $^{14}$CO$_2$ on various days after pollination. Variation in these parameters may be the result of the presence or absence of invertase in wildtype and \mn1 phenotype kernels, respectively. Invertase influences sugar composition in maize kernels tissues and the differences may be the consequence of a sugar-sensing signal transduction process.
2.1 Plant Materials

The homozygous wildtype inbred used in this study was Wisconsin 22 (W22) and the homozygous miniature inbred was in W22 genetic background. The original seeds were obtained from Dr. P. Chourey of the University of Florida. Succeeding generations were produced at the Pennsylvania State University.

2.1.1 Growth condition

Most of the plants used in the studies were grown in the greenhouse in 20 liter plastic pots covered with aluminum foil to shield the pot from direct sunlight and filled with a low density growing media that included 2 parts peat, 2 parts perlite and 1 part silty clay loam soil. High pressure, high intensity discharge (HID) sodium vapor lamps were used to supplement daylight and maintain a 18/6 hour light/dark pattern throughout the year. Exhaust ventilation and evaporative cooling pads helped to control the greenhouse temperature between 26.6 and 18.3 °C for day and night, respectively, during most growth periods. On hot summer days, greenhouse air temperature tended to track outside air temperature when these temperatures exceeded the set temperature of 26.6 °C. Plants for the cytokinin study were grown in the field at the Russell E. Larson Agricultural Research Center of the Pennsylvania State University from May to August 1996.
2.1.2 Split-pollination

In previous studies, homozygous wildtype and 

\textit{mnl} mutant plants were used to

study assimilate partitioning mechanisms in maize kernels (Shannon, 1968; 1972; Porter
et al., 1985; 1986; 1987a; Shannon et al., 1993). A common problem with comparing

assimilate transport and partitioning between individual homozygous wildtype or mutant
genotypes is the plant to plant variability. A split-pollination procedure was developed to

establish two kernel populations with different endosperm genotypes on a single ear. The

utility of split-pollination for studying assimilate partitioning in maize kernels is based on

the knowledge that endosperm is a triploid tissue and on results of previous cross pollination studies between wildtype and \textit{mnl} genotypes (Miller and Chourey, 1992;
Cheng et al., 1996). Heterozygous wildtype kernels on homozygous \textit{mnl} plants develop

a homozygous wildtype phenotype. In my experiments with split-pollination plants,
homozygous \textit{mnl} maize plant is used as the maternal parent. All ear shoots of the
maternal plant were covered prior to silk emergence to restrict natural pollination. Silks
of the uppermost ear shoot that were ready for pollination were clipped 1 to 2 cm below
the top of the husk 2 to 3 days after first silk emergence and re-covered. The residual
silks extruded about 2 cm in the 24 hours following clipping. They were divided into two
equal halves and each half was covered with a 6.5 x 11 cm paper coin envelope. Half of
the silks were hand pollinated with pollen from homozygous wildtype plants and re-
covered. The procedure was repeated on the other silks, but with \textit{mnl} pollen. The two
portions of silks were covered separately to avoid pollen contamination between them.
The envelopes were marked with the pollen source and the ear shoot with coin envelopes
in place was covered with an ear shoot bag. With this pollination scheme, kernels on the
self or sib pollinated side of the ear have two copies of the \textit{mnl} gene from the central cell
(maternal plant) and one more copy of the \textit{mnl} gene from the sperm nucleus (pollen) in
their endosperm cells. The endosperm genotype of the homozygous kernels is

\textit{mnlmn1mn1}, and its phenotype is that of a homozygous \textit{mnl} plant. The heterozygous
kernels on the side of the ear shoot pollinated with homozygous wildtype pollen produce
endosperms of genotype \textit{Mnlmn1mn1} but with a kernel phenotype indistinguishable from
homozygous wildtype kernels. Use of this split-pollination procedure produces kernels of two phenotypes on one single ear. Comparison of assimilate transport processes between kernels of the two genotypes reduces the problems associated with plant to plant variance. For convenience, kernels with \textit{Mn1mn1mn1} and \textit{mn1mn1mn1} endosperm genotype on split-pollinated plants are called \textit{wt} and \textit{mn} kernels, respectively.

2.2 Methods

2.2.1 Assimilate partitioning study

Two experiments were conducted to study assimilate partitioning and compartmentation during development and growth of maize kernels. In the first experiment self-pollinated wildtype and \textit{mn1} plants were used whereas split-pollinated plants were used in a follow up experiment. Four self-pollinated \textit{mn1} and two self-pollinated inbred wildtype plants were used for the carbohydrate compartmentation study at 10, 13, 16, and 22 DAP. The self-pollinated homozygous \textit{mn1} and wildtype plants were grown at the same period of time in the greenhouse in a split-split plot design with four or two (\textit{mn1} and wildtype, respectively) replications over time. The whole plot treatment was genotype, the split-plot was days after pollination (10, 13, 16, and 22 DAP), and the split-split plot treatment was kernel harvest at 2, 6, and 12 hours after pulse labeling plant leaf tissue with $^{14}$CO$_2$. In the second experiment with split-pollinated plants, a split-split plot design was used with a whole plot of 10 or 21 days after pollination and 5 replications over time. A split plot of endosperm genotype (\textit{mn1mn1mn1} and \textit{Mn1mn1mn1}) and a split-split plot of kernel harvest at 3, 6, and 9 hours after $^{14}$CO$_2$ labeling pulse leaves. Data obtained from the first experiment with self-pollinated plants was analyzed with the SAS GLM program and those from the second experiment with split-pollinated plants with SAS Proc Mixed program (SAS Institute, Inc., Cary, NC).
Three split-pollinated plants with ears at 21 DAP were used for the agar influx study and one split-pollinated plant with an ear at 10 DAP was used for the assimilate transport kinetics study.

2.2.1.1 Kernel-cup technique

A kernel-cup technique modified from Porter et al. (1985) was applied to kernels of split-pollinated plants in the agar influx experiment before the plants were labeled with \(^{14}\)CO\(_2\). Several rows of kernels on an attached ear were exposed by peeling back the husks on one side of the ear. The distal halves of several kernels were surgically removed with a scalpel and the endosperm tissue was subsequently removed with a sharpened spatula such that each kernel base with attached pericarp formed a cup which remained attached to the ear. These kernel cups consisted of pedicel parenchyma with embedded vascular and attached pericarp. Porter et al. (1985) showed that assimilates continue to enter the agar replacement of the endosperm from the plant source for up to 8 hours. Experimental units consisted of groups of three kernels together in a single row while replications usually consisted of similar groups on separate kernel rows. Each pedicel cup was briefly rinsed with water and the excess water was removed with micropipette. Seventy µL of warm (38 °C) 1% agar solution made in 20 mM MOPS buffer (pH 7.0) was added to each kernel cup of one 21 DAP split-pollinated plant. The other two 21 DAP split-pollinated plants were treated the same except that 0.01 mM PCMBS was added to the agar solution. The solidification of the agar took about 15 minutes. Husks were closed back over the ear to avoid water loss. Subsequently the plants were fed with \(^{14}\)CO\(_2\) as described in the pulse-chase procedure.
2.2.1.2 $^{14}$CO$_2$ administration

The $^{14}$CO$_2$ pulse-chase procedure used in split-pollinated plants was modified from Shannon (1968). Briefly, the top half of the plant (including the ear) was enclosed in a large transparent plastic bag (100 x 85 cm). The $^{14}$CO$_2$ generator consisted of a 15 mL glass vial with 17 mg Barium Carbonate-$^{14}$C containing 100 $\mu$Ci and fitted with a 3-hole rubber stopper. One tygon tube (1 cm i.d.) whose end was taped to the top of plant was connected to one hole of the stopper. A return tube, containing a rubber bulb unidirectional pump, was taped to the plant near the bottom of the bag and connected to the second hole in the stopper. A short section of tygon tubing fitted with a screw clamp was connected to the third hole. A foam plastic gasket (30 cm x 10 cm x 2 cm) was wrapped around the plant stem at the point of attachment of the plastic bag. Portions of the inlet and return tubes were sandwiched between two layers of the gasket and tightly tied with string. Immediately before treatment the plastic bag was placed over the plant and tied at the gasket. Three mL of lactic acid were added to a vial containing Ba$^{14}$CO$_3$ through the short tube. The liberated $^{14}$CO$_2$ was swept into the plastic bag by repeated compression of the rubber bulb pump and stirred by manually compressing and expanding the bag, creating internal turbulence. After 30 min of photosynthesis in the presence of $^{14}$CO$_2$, the bag was removed and the plant allowed to continue normal growth for the chase periods. In the study with self-pollinated plants, a LI-COR (LI-COR Inc., Lincoln, NE.) Model 6200 photosynthesis system was used to expose the second leaf above the ear leaf to $^{14}$CO$_2$ for 15 min. A vial containing Ba$^{14}$CO$_3$ was placed in the 1-L LI-COR leaf chamber and closed over the target leaf. With the system operating in closed photosynthesis measurement mode, 3 mL of lactic acid was injected into the vial to release $^{14}$CO$_2$. After 15 min., residual $^{14}$CO$_2$ was scrubbed from the leaf chamber and the chamber was removed to begin the chase period.
2.2.1.3 Sample collection

In the assimilate partitioning study with self-pollinated plants, the distal, middle and basal third of the ear attached to the plant was removed at 2 (tip), 6 (Middle), and 12 (basal) hours, respectively, after $^{14}$CO$_2$ labeling at each of four kernel growth stages (10, 13, 16 and 22 DAP). Twenty kernels were removed from each ear portion and separated into pedicel, chalazal, endosperm, and at 16 and 22 DAP, embryo tissue. Pericarp tissue was discarded. All the tissues were frozen on dry ice, freeze-dried, weighed. Sugars and starch were extracted from the tissues and measured and their radioactivity determined.

In the assimilate partitioning study with split-pollinated plants, the distal, middle and basal third of the ear attached to the plant was removed at 3, 6, 9 hours, respectively, after $^{14}$CO$_2$ labeling at 10 and 21 DAP. Ten kernels of each genotype were excised from ear piece at each sampling time. Endosperms were excised from the kernels and freeze-dried. Dry weight of the endosperms was measured and the amount and radioactivity of sugars in the endosperms were determined.

Kernels from one split-pollinated plant at 10 DAP were used in the assimilate transport kinetics study. Half the ear was removed at 3 and 6 hours after $^{14}$CO$_2$ labeling. Twenty kernels of each genotype were excised from the ear at each time. Ten of them were separated into pedicel, P-C tissue, and endosperm while the other 10 kernels were left intact. All tissues were frozen, freeze-dried, and weighed.

In the agar influx experiment, 3 agar traps and 3 intact kernels were randomly collected from split-pollinated plants at 21 DAP at 20 minute intervals from 140 to 240 minutes after labeling with $^{14}$CO$_2$ in about. Endosperms were excised from the kernels. The 3 agar traps or 3 endosperms were combined as one sampling unit in radioactivity measurement.
2.2.1.4 Sample extraction and measurement

When needed, the samples were freeze-dried with Lanconco freeze-dry system (LYPH-LOCK12). The extraction buffer was MCW (methanol:chloroform:water, 13:4:3, v/v) except where specified. A Tissue Tearor (Model 985-370, Biospec Products, Inc.) was used when tissues need to be homogenized. Ecoscint liquid scintillation fluid (Pational Diagnostics Instrumentation Chemicals, Manville, NJ) was used in radioactivity measurement which was counted with a Packard 1500 liquid scintillation analyzer (Packard, Downers Grove, IL).

The agar traps and the endosperms of the agar influx study as well as the tissue parts of the kinetics study were extracted with 1.5 mL MCW overnight before homogenization for 1 min. The homogenate was centrifuged at 4,000g for 5 min and the pellets were extracted twice more with 0.5 mL MCW. The supernatants of the three extractions were pooled together and used to measure radioactivity.

Except for the experiments described in the previous paragraph, the same extraction procedure was applied to all the freeze-dried tissue parts from split-pollinated and self-pollinated plants. Freeze-dried tissues were homogenized in 5 mL MCW. The samples were centrifuged at 3,500g for 10 min and the supernatant collected. The extraction procedure was repeated two more times using the pellet and the pooled supernatants were frozen, freeze-dried and re-dissolved in 500 µL water. Sugar components in the samples were separated by paper chromatography. Briefly, a strip of Whatman 3 MM chromatographic paper was cut into 4 cm by 55 cm strip and marked at 5 and 10 cm starting from the upper edge. The samples were applied as a narrow streak along the 10 cm line in 25 µL doses after allowing drying of the previous application. The descending chromatogram was developed over 48 hours in a monophasic solvent system (butanol:acetic acid:water, 3:1:1, v/v). The sugar zone was located by placing the chromatograms in a plastic template and spraying a 1 mm wide streak down the center of the paper strips with the an aniline-diphenylamine-phosphate spray reagent (2% aniline in
acetone, 2% diphenylamine in acetone, and concentrated phosphoric acid; 5:5:1, v/v). The paper strips are cut into rectangular pieces containing sucrose, glucose and fructose, respectively. Each piece was folded and put into a 50 mL glass scintillation vial with omni-scint toluene to count radioactivity. The paper pieces were removed from the counting vial, rinsed with toluene and air dried. Sugar on the paper was extracted with 3 mL of water. Glucose and fructose were measured according to Nelson (1944). Briefly, to a 1 mL aqueous sugar solution having not more than 120 µg sugar, add 1mL of reagent A [low alkalinity copper reagent; 12 g potassium sodium tartrate and 24 g anhydrous Na₂CO₃ are dissolved in 250 mL water. CuSO₄-5H₂O (4 g) is dissolved in 20 mL water and added. NaHCO₃ (16 g) is added to the above solution in small amount while stirring. Na₂SO₄ (180 g) is added to 500 mL water and boiled to expel air. The two solutions are combined and diluted to 1 L with H₂O. Allow the solution to stand for one week and use the clear supernatant]. Heat all the samples, blanks and standards for 10 min in boiling water bath. After cooling in ice water, 1 mL of reagent B [arsenomolybdate reagent; 25 g Ammonium molybate is dissolved in 450 mL H₂O. Add 21 mL 96% H₂SO₄ is added to above solution. Disodium-hydrogen arsenate heptohydrate (Na₂HAsO₄·7H₂O, 3.0 g) is dissolved in 25 mL H₂O then add to above solution. Incubate for 24 hours at 37°C store in glass stoppered brown bottles] was added. The tubes were stirred immediately before 7 mL water was added. The stirred tubes were held for 15 to 40 min before reading color intensity at 520 nm in a split-beam Spectronic 1001 spectrophotometer (Milton Roy Company). Sucrose was measured by the Anthrone reaction. Anthrone reagent containing 86 mL concentrated H₂SO₄ (13.8N), 20 mL H₂O and 0.15 g Anthrone was prepared fresh and kept cold for use within two days. One mL of anthrone reagent was added to a 10 mL glass centrifuge tube containing a 50 µL samples aliquot. The tubes were stirred immediately and incubated at 45°C for 15 min for complete color development. The color intensity was read in the split-beam spectrophotometer at 620 nm within 30 min.

After the MCW extraction and final centrifugation, the pellets were used for starch analysis. The pellets were resuspended in 0.5 mL of water and boiled for 5 min.
Another 0.5 mL of water was added and the pellets were boiled again. Samples were incubated at 30°C for 24 hours with 100 µL of enzyme solution [0.5 M sodium acetate (pH 4.8) containing 5 units of amylglucosidase and 2.5 units of amylase]. The samples were centrifuged at 4,000g for 5 min. The content and radioactivity of the glucose released by the enzyme in the supernatants were measured as described previously.

2.2.2 Biomass measurement

At 6, 9, 12, 16, and 21 DAP, 10 kernels of each genotype were collected from the same attached split-pollinated ears on five replicate plants. Five kernels were separated into pedicel, P-C, endosperm, and embryo (16 and 21 DAP samples) tissue. Fresh weights of intact kernels and tissue parts were measured. The samples were dried at 60°C for 48 hours, weighed, and held for biochemical or radioactivity analysis.

2.2.3 Histochemical detection of invertase activity

A procedure modified from Doehlert and Felker (1987) was followed for histochemical detection of invertase. After a specified period of growth, both wt and mn kernels were excised from split-pollinated ears. The kernels were hand sectioned longitudinally and fixed in 4% Formalin (pH 7.0) for 30 min. The sections were then rinsed in several changes of distilled water over the next few hours to remove all endogenous sugars. Following rinsing, the sections were incubated in the dark at room temperature in a reaction mixture comprised of equal volumes of 0.56 mg/mL phenazine methosulfate, 0.96 mg/mL nitro blue tetrazolium, 0.067 mg/mL glucose oxidase (119,000 units/gram solid), and 20 mg/mL 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 postfixing in 4% Formalin for 15 min. Sections were rinsed several times with distilled water and stored in 15% ethanol at 4°C.
2.2.4 Sampling for cell number, starch granule number and endoreduplication

The *mn* and *wt* kernels from the middle section of split-pollinated ears were excised and immediately placed in ethanol:glacial acetic acid (3:1, v/v). After at least 24 hours, the kernels were sequentially equilibrated in 80, 50, 25 % ethanol (v/v) and water. The endosperms were excised and digested following the procedure of Myers et al. (1990). A pectinase solution for endosperm digestion was prepared by mixing 1 part pectinase (technical grade with inert filler, No. 10258, ICN Biomedicals, Cleveland, OH) to 3 parts citrate-phosphate buffer [8.8 g/L Na₂HPO₄, 3.6 g/L citric acid (pH 4.0), 0.1% NaN₃ (w/v)] and filtered twice through Whatman No. 1 paper to remove the inert filler. Excised endosperms were placed in 1 mL of pectinase solution in a tightly capped tube, and incubated at 40 °C until soft. Endosperms less than 10 DAP required approximately 12 to 24 hours in the pectinase solution; older endosperms required up to 48 hours to soften. Nuclei and starch granules were dispersed by forcing the digested tissue through a 20 gauge needle with a syringe. An aliquot was removed from the suspension and diluted with an equal volume of an iodine solution (3.3 g/L I₂ + 6.7 g/L KI) to stain the starch granules. Stained starch granules were counted on a hemacytometer (Improved Neubauer Ultra Plane, model 3500, Hausser Scientific, Blue Bell, PA) at 400X magnification. The counts were multiplied by the appropriate dilution factors to determine starch granule number per endosperm. An aliquot of the suspension was used to measure nuclei number and nucleus DNA endoreduplication with a flow cytometer. The flow cytometry procedure can be described briefly as follows. A 150 µL sample aliquot was mixed with 120 µL Flow-Check® Fluorospheres (Coulter Corporation, Miami, FL, 1.25x10⁶ per mL), 5 µL fluorescence dye Yo-Pro® Iodide (a DNA-binding fluorochrome, Molecular Probes, Eugene, OR) dissolved in DMSO, 3 µL Chicken Erythrocyte Nuclei Singlets (BioSure Controls, Grass Valley, CA) and 722 µL Isoton buffer (Coulter Corporation, Miami, FL) in 5 mL plastic centrifuge tubes. Samples were analyzed on a XL-MCL benchtop cytometer (Beckman Coulter, Miami, FL) equipped with a 15mW air-cooled 488nm argon-ion laser. Log green Yo-Pro fluorescence (nuclei) was collected after filtering through 530/30-nm band-pass filter. Log red fluorescence
(Fluorospheres) was measured using a 610/30nm band-pass filter. A minimum of 10,000 events were counted after forward and side scatter gating to remove debris and clumps.

2.2.5 Enzyme activity measurement

The enzyme preparations and all the standards were purchased from Sigma Chemical Co. One unit of enzyme was defined as an amount that converts 1 µmole of substrate to product per min under optimal conditions of measurement. A Zeiss PMQ II Spectrophotometer (Carl Zeiss Inc, Oak Brook, IL) connected to an ISCO Model 170 Chart recorder (Instrumentation Specialties Company Inc., Lincoln, NB) was used for AGPase activity and all other absorbency was measured with a split-beam spectrophotometer (Spectronic 1001, Milton Roy Company). The scintillation fluid used in radioactivity measurement was Ecoscint (Pational Diagnostics Instrumentation chemicals, Manville, NJ). The radioactivity was counted with a Packard 1500 liquid scintillation analyzer (Packard, Downers Grove, IL).

2.2.5.1 Sample preparation

Split-pollinated ears were excised from the plants and immediately frozen in liquid nitrogen. The ears were freeze-dried after scoring the top of each kernel to fracture the pericarp. Both mn and wt kernels were removed from each ear and their endosperms were excised. Fifty milligrams of freeze-dried and pulverized endosperm tissue were added to a 15 mL plastic centrifuge tube containing 2 mL of ice cold Hepes grinding buffer [50 mM Hepes, (pH 7.5), 0.5 M Sorbitol, 10 mM KCl, 1 mM MgCl$_2$.6H$_2$O, 1 mM EDTA, 0.1% BSA, and 5 mM DTE]. Samples were kept on ice while sonicated for four 10-second bursts with a 10-second rest period between each burst using the Biosonic IIA ultrasonic probe set at 60% maximum power. The homogenate was centrifuged at 3,500g for 10 min at 4 °C. The supernatant was filtered through one layer of Miracloth and kept cold for enzyme assays.
Measurement of AGPase activity needs freshly prepared endosperm extracts. Measurement of sucrose synthase, starch synthase, and starch branching enzyme activities can be determined by using the same endosperm extract prepared for AGPase analysis, but only after sugars are removed from the endosperm extracts. Two mL G-25 columns were used for removing sugars from the enzyme extracts. These G-25 columns were prepared with pre-equilibrated Sephadex G-25. Sephadex G-25 was kept in the equilibration buffer [50 mM MOPS, pH 7.5 (20.93 g/L), 15 mM MgCl$_2$·6H$_2$O (4.078 g/L), 1 mM EDTA (0.744 g/L), 2.5 mM DTT (0.386 g/L)] overnight before usage. The prepared columns were placed on top of 15 mL plastic centrifuge tubes and spun at 1,000g for 4 min. The volume of G-25 in each column was adjusted to exactly 2 mL and the solvent in the centrifuge tubes was decanted. A 0.5 mL aliquot of endosperm extract was added gently on the top of the columns. The apparatus was spun at 1,000g for 1 min and the de-sugared endosperm extract was collected in the centrifuge tube.

2.2.5.2 AGPase assay

The procedure described by Echeverria et al. (1988) was followed for assaying AGPase. Briefly, the assay buffer composed of 50 mM Heps, pH 8.0 (1.0 M, 50 µL), 5 mM MgCl$_2$ (0.1 M, 50 µL), 0.05% BSA (1%, 50 µL), 0.65 mM PPI (13 mM, 50 µL), 0.2 mM NADP (4mM, 50 µL), 5 units of G6PDHase, 5 units of PGM, and 0.4 mM ADPG was added to 50 µL of endosperm extract. The numbers in parentheses are concentration and volume of stock solution used in each assay. The final volume was made up to 1 mL by adding water. The assay was carried out in a 1 mL cuvette. The amount of NADPH produced by the AGPase reaction was measured in the split-beam spectrophotometer at 340 nm.

When endosperm extracts were ready for AGPase measurement, all reaction mixture ingredients were added to the 1 mL spectrophotometer cuvette except
G6PDHase, PGM and ADP-glucose. G6PDHase was added to the mixture first to metabolize any G6P present in the endosperm extracts. After the G6P was metabolized, PGM was added to metabolize any G1P. When no further G1P activity was observed, ADP-glucose was added. The net change of O.D. per minute divided by 6.22 describes the µmol of the substrate consumed per minute by enzymes from the endosperm extract in the cuvette.

2.2.5.3 Sucrose synthase assay

Sucrose synthase activity was determined according to Shannon et al. (1996). The assay was conducted in 1.7 mL micro-centrifuge tubes. A 45 µL aliquot of desugared endosperm extract was added to each tube along with 25 µL of a sucrose synthase substrate mixture [50 mM MOPS, pH 7.5 (10.465 g/L), 15 mM MgCl2-6H2O (3.05 g/L), 28 mM D-Fructose (5.044 g/L) and 18 mg/mL UDPG (added to the substrate mixture right before usage)] and incubated at 25°C for exactly 10 min. The reaction was stopped by adding 70 µL of 30% KOH to each sample and heated in a boiling water bath to decompose the unreacted fructose. For the 0 hour control, 70 µL of 30% KOH was added to the tube before adding the endosperm extract and the substrate mixture. Sucrose produced by the assay was measured by the Anthrone method (detailed in assimilate partitioning study).

2.2.5.4 Starch synthase assay

The starch synthase procedure described by Echeverria et al. (1988) was followed. Two sets of 40 uL aliquots of de-sugared endosperm extracts were prepared for assay. One set kept in boiling water for 90 seconds served as controls. A reaction mixture [20 µL 1 M Bicine (pH 8.5), 10 µL 0.25 M EDTA, 10 µL 0.2 M GSH (Glutathione, 61.4 mg/mL), 10 µL 1 M potassium acetate, 50 µL 20 mg/mL RLG (Rabbit
Liver Glycogen), 50 µL 20 mM $^{14}$C-ADPG, 40 µL 1.25 M Sorbitol] was added to samples and controls. The mixtures were incubated at 30°C for 30 min. The reaction was stopped by putting the mixtures in boiling water for 90 seconds. Two mL of 1% KCl-75% methanol was added to each assay tube and tubes were kept on ice for 10 min. The samples were centrifuged at 1000g for 5 min and the supernatants were decanted into the radioactive waste container. The pellets were resuspended in 0.2 mL water and the KCl-methanol procedure was repeated two more times. The final pellets were dissolved in 1 mL water and half of the volume was used to count the radioactivity after adding 5 mL scintillation fluid.

2.2.5.5 Starch branching enzyme assay

The activity of starch branching enzyme was determined according to Shannon et al. (1996) and was based on the stimulation of Phosphorylase-a by branching enzyme. A 20 µL aliquot of endosperm extract was added to an assay mixture [10 µL phosphorylase-a (2.5 mg/L H$_2$O, freshly made), 10 µL 1 M Na Citrate (pH 7.0), 30 µL 0.15 M G1P, 10 µL 10 mM AMP (pH 7.0, but needs high pH to dissolve), and 20 µL H$_2$O] and incubated at 30°C for 60 minutes. Samples without Phosphorylase-a were used as controls. The assay reaction was stopped by immersing tubes in boiling water for 90 seconds before a proper dilution of reaction mixture with water (usually 5 mL) was made. The released inorganic phosphate was measured with Malachite Green method. The Malachite Green assay solvent includes 0.045% malachite green hydrochloride (MG, dissolved in water, 0.45 mg/mL), 4.2% ammonium molybdate in 4N HCl (AM), 0.02% Sterox SE (ST) and 34% sodium citrate. MG and AM (3:1, v/v) were mixed for at least 20 min prior to their use and filtered through Whatman No.5 filter paper. One hundred µL ST was added to the filtered mixture for every 5 mL MG/AM. The assay mixture contains 50 µL starch branching enzyme reaction mixture and 800 µL MG/AM/ST. One hundred µL citrate solution was added 1 min later. Color reaction was read immediately with a split-beam spectrophotometer at 660 nm using a 1 mL cuvette. Color is stable at least 4 hours.
2.2.6 Hormone measurement

After a given period of growth, split-pollinated ears were excised from the plants and kept on ice. About 500 mg of endosperm tissue and 100 mg of embryo tissue (about 2 to 16 endosperms and 6-27 embryos, depending on genotype and developmental stage) from both *mn* and *wt* kernels on the same ear were excised and kept in pre-weighed vials containing extraction solution (W1). The vials with excised tissue were weighed (W2) and tissue fresh weight was obtained by subtraction (W2-W1). Three replicates of each sample were collected. Samples for ABA and IAA measurement were prepared and measured with ELISA as described by Ober et al. (1991) and Lur and Setter (1993a), respectively. Samples for cytokinins were prepared and measured with HPLC according to Jones et al. (1990). Radioactive ABA, IAA and cytokinin standards were used as an internal standard to monitor the extraction system and determine the hormone recovery rate.

2.2.6.1 Sample preparation for ABA and IAA assay

The procedure detailed in Ober et al. (1991) was followed for the assay. Endosperm tissue samples were kept in capped 1.5 mL micro-centrifuge tube with 0.5 mL extraction buffer [80% methanol, 1% glacial acetic acid (v/v) and 10 mg/L Butylated Hydroxytoluene (BHT)] at -80°C for up to 6 months between collection and analysis. The sample tissues in the extraction buffer were transferred to 10 mL centrifuge tubes. Two mL of extraction buffer was used to rinse the micro-centrifuge tubes and the washes were combined with the samples. The sample tissues were homogenized (Brinkmann Instruments, PT10/35) at power level 5 for two 10-second pulses with a 10-second pause in between. One mL of extraction solvent was used to rinse the probe after homogenization and the wash was pooled with the sample. Samples were centrifuged at 1,000g for 5 min at 4°C. One mL of supernatant was transferred to a 1.7 mL micro-
centrifuge tubes and dried in vacuo. Samples were resuspended in 0.2 mL 1% glacial acetic acid (v/v). A 0.1 mL aliquot of resuspended extract was loaded onto a C18 reverse-phase chromatography column (4 mm i.d.) containing 0.6 gram of 40 µm particle size packing material (bonded-phase octadecylsilane, J.T. Baker Chemicals, Phillipsburg, NJ) which had been pre-equilibrated with solvent A [20% methanol (v/v), 1% glacial acetic acid (v/v), and about 0.16% (v/v) triethylamine (pH 3.25)]. In sequence, the column was eluted with 1.5 mL of solvent A, 1.5 mL of solvent B [30% methanol (v/v), 1% glacial acetic acid (v/v)] to extract IAA and 1 mL of solvent C [55% methanol (v/v), 1% glacial acetic acid (v/v)] to extract ABA. The ABA eluate was used directly in the ELISA and the IAA eluate was methylated with diazomethane (Lur and Setter, 1993a) before analysis with ELISA.

2.2.6.2 Indirect ELISA method for ABA and IAA determination

Round-bottom 96-well microtiter plates (Corning High-binding #25802 plates) were used for ELISA-ABA. Plates were coated with 200 µL/well of well-mixed ABA-BSA conjugate solution which contains 200 µL of conjugate stock solution (a 100X solution contains 0.7 mg protein/mL in 50% glycerol/bicarbonate buffer stored at -20°C) and 21 mL bicarbonate/carbonate buffer (B/C) [50 mM NaHCO₃ (pH 9.6) and 0.02% sodium azide]. The plate was covered with a rigid-plastic cover and placed into a plastic bag, sealed, and held in a refrigerator (4°C) overnight. The coating buffer was discarded and the plate was blotted on a paper towel. The plate was rinsed with tris-buffered saline (TBS) [50 mM Tris (pH 7.5), 1 mM MgCl₂, 10 mM NaCl, 0.02% NaN₃] four to six times for 5 min duration for each rinse. At the sixth rinse, TBS with tween-20 (TBST) [50 mM Tris (pH 7.5), 1 mM MgCl₂, 10 mM NaCl, 0.02% NaN₃, 0.1% tween-20] was used to make sure that all loosely attached ABA-BSA is removed. One hundred µL of hepes-buffered saline (HBS) [50 mM Hepes (pH 7.5), 1 mM MgCl₂, 10 mM NaCl, 0.02% NaN₃] was added to each well of the plate. A standard dilution series of ABA was prepared fresh in duplicate from a common stock solution in rows C and F of each plate.
Hepes buffered saline with methanol (HBSM) [40 mL of 5X TBS + 10 mL methanol + 150 mL H$_2$O] was used for dilution and the final amount (+)ABA in the wells ranged from 0.06 to 0.2 pmol per well. Aliquots (typically 10 µL) of endosperm extract (usually duplicate wells for each sample) were added to other appropriate wells. One hundred µL of primary monoclonal antibody (final amount is 100 µg/well) was added to each well. Primary antibody stock solution (100 X) is 2 mg PHYTODETEK mAb + 10 mL TBS + 10 mL glycerol stored at -20°C in small aliquots. This makes a final dilution of 1 mg protein/mL. The plates were sealed with a rigid cover and outer plastic bag and incubated overnight in a refrigerator at 4°C. After the plate was repeatedly washed with TBST for a total of four times with a 5-min incubation period each time, 180 µL of secondary antibody (anti-mouse IgG-alkaline phosphatase conjugate, Sigma product A-3562) was added to each well. The plate was incubated and washed as described after primary antibody. The final substrate, 180 µL para-nitrophenylphosphate (PNPP) reagent pre-mixed with diethanolamine (DEA) buffer [1 mg/mL PNPP, 0.9 M DEA, 3 mM MgCl$_2$, pH 9.8] was added to each well and the plate was incubated at room temperature until proper color developed, normally 2 to 4 hours. An air dried well (usually A1) was used as a blank for the colorimetric assay. The plate was read with a plate reader (Model 2550, Bio-Rad) at 405 nm and data were analyzed with an automated data analysis (spreadsheet macro file ELISA_ro.XLS, copyright by Dr. Tim Setter of Cornell University). Standard curves were plotted with the readings in Row C and F with ABA standards. The regressions of the standard curves were determined and sample amounts in each well were calculated.

The methylated and dried IAA was resuspended in 50 µL of 100% methanol and used for the ELISA. For ELISA-IAA, all the procedures are similar to ELISA-ABA but the plate was coated with IAA-BSA instead of ABA-BSA. Substrate PNPP was used instead of using a secondary antibody.
2.2.6.3 Cytokinin extraction and measurement

The method of Jones et al. (1990) was followed for cytokinin extraction and measurement. Ten µL $^3$H-ZR was added labeled corex tubes and two vials used as an internal standard. The radioactivity in the two vials was read with 40 mL scintillation fluid (Ecoscint, Pational Diagnostics Instrumentation Chemicals, Manville, NJ). The average dpm of these two vials was used as this batch sample original count of radioactivity in recovery rate adjustment. Endosperm samples of approximately 0.6 to 1.0 g fresh weight [held in approximately 5 mL extraction buffer (methanol : water : acetic acid (70:30:3, v/v) with 10 mg/L butylated hydroxytoluene) per gram fresh weight of tissue at -80°C] were transferred into the above corex tubes and placed on ice. The sample storage vials were rinsed with 1-2 mL cold extraction buffer and the rinse solution was added to the corex tubes. The samples were homogenized with a Polytron homogenizer (model PT10/35, Brinkman Instruments, Inc., Westbury, NY) in a -80° C extraction buffer for 30 seconds at the power level of 8. During homogenization the corex tubes were jacketed with a Styrofoam vessel containing 95% ethanol and several pieces of dry ice to maintain -80°C condition in the jacket solution. Tissue pieces adhering to the homogenizer probe were dislodged with dental needle and the sample was homogenized for another 10 seconds. The probe was flushed with ice cold extraction buffer and the washes were pooled with the tissue and extract buffer. After homogenizing each sample, the probe was washed with 15 mL methanol for 10 seconds once and twice with 15 mL water for 10 seconds. These washings were discarded. The vials were centrifuged at 5,000 g for 25 min at 0°C. The supernatants were poured into labeled vials held at 4°C and the pellet was held on ice. The pellets were resuspended with 2-3 mL cold extraction buffer and centrifuged again at 5,000g for 25 min at 0°C. The supernatants of the two centrifugations were pooled. The samples were dried in vacuo overnight and held in -80°C. One mL anion exchange columns were prepared with 2 mL of pre-equilibrated DEAE-Sephadex:DEAE-Cellulose (2:1) anion exchange packing material [1:1 diluted with 10 mM Bis-Tris (BT) buffer, pH 6.5]. Five mL of BT buffer was used to rinse the columns after the initial packing material drained and the
columns were moved to a cold room (4 °C). In the cold room, 2-3 mL 10 mM BT buffer was used to resuspend the dry pellets and the samples were sonicated in an ultrasonic cleaner (Branson B-220, Shelton, CT) for 10 min. The samples were transferred to labeled columns. The sample vials were rinsed twice with 2 mL BT buffer and the buffer rinsate was added to the columns. Lastly, 2 mL of BT buffer was added to each column to enhance recovery. The collected eluates were applied to 20 mL antibody columns (anti-cytokinin immunoaffinity column, pre-equilibrated with 10 mL BT buffer). The sample vials were rinsed twice with 2 mL of BT buffer and the rinsate was applied to the columns. Five mL of BT buffer was added to each column to elute the nonspecific binding compounds and 10 mL of water was added to each column to elute the salts. Ten mL of HPLC grade methanol was added to each column and the eluate was collected in labeled vials. The samples was dried in vacuo and held at -80 °C. Five hundred µL kinetin (0.4 mg/L) was added to each sample vial and the vials were sonicated for 5 min at room temperature. The solution was transferred to a Microspin filter with 0.2 µm Nylon Non-ST (Chrom Tech, Apple Valley, MN). Another 100 µL of kinetin solution was added to each vial and pooled into the filter. The microspin filters were centrifuged at 2,500g for 10 min. The samples, in a final volume of 600 µL, were held for HPLC analysis. Separation and quantification of cytokinins were achieved via HPLC (C18, Spherisorb column) with a diode array detector. Authentic cytokinin standard were used to construct standard curves. Values were adjusted for assay losses based on the recovery of ³H-ZR added to sample tissue prior to extraction.
Chapter 3

ASSIMILATE TRANSPORT INTO MAIZE KERNEL

3.1 Introduction

Assimilates are routinely transported from source to sink tissue during growth and development of maize. During most of its reproductive period, maize kernels constitute the major sink. Dry matter production in maize kernels is controlled by several complex physiological processes including photosynthetic rate, phloem loading, long distance transport, phloem unloading, movement of assimilates into kernel endosperm tissue, and storage of sugars or their utilization in starch synthesis (Felker and Shannon, 1980). Each of these processes is comprised of many metabolic steps and controlled by numerous regulatory factors. The understanding of assimilate partitioning and the mechanisms that control it in maize is of great importance not only for maize physiological study but also for its application in the agricultural industry.

Previous studies have shown that sucrose is the main assimilate form transported in the maize plant (Shannon, 1972; Prioul, 1996). The transported sucrose undergoes phloem loading, long distance transport, phloem unloading and phloem post-unloading to reach storage sites in kernels. Driving forces are needed for long distance transport of assimilate from sources to sinks and for short distance assimilate allocation within sink tissue in order to maintain assimilate transport to sinks. As early as 1930, Münch suggested a model that postulates that the loading, transport, and unloading from the conducting tissue are mainly driven by concentration and/or osmotic gradients (Herbers and Sonnewald, 1998). According to the pressure-flow theory, the mass flow of sucrose and water along the sieve tubes is driven by the turgor pressure difference within the
phloem from source to sink. Eventually sucrose reaches kernel pedicel tissue where it is unloaded passively from the pedicel symplast (Shannon, 1968, Porter et al., 1986). Gross autoradiography of kernel tissue shows that labeled assimilates accumulate within the pedicel prior to their slow movement into the endosperm and that a large portion of the sucrose is hydrolyzed to hexoses during the transfer process (Shannon, 1972). Since there are no vascular or plasmodesmatal connections between the maternal tissues of the pedicel and the developing endosperm cells in maize, assimilates must follow an apoplastic pathway to move from pedicel to endosperm tissue (Felker and Shannon, 1980). That assimilates are taken up from the pedicel apoplast free space prior to their utilization within the developing endosperm or embryo and that apoplastic solute concentration has a regulatory role in the post-unloading process are supported by agar influx studies of Porter et al. (1985; 1987a; 1987b). Apoplastic solute content could be dependent on rate of unloading from the maternal tissue and/or rate of absorption by the developing endosperm and embryo. Porter et al. (1987a) provided evidence that assimilate transport into the endosperm is regulated by endosperm cell turgor. Sugar uptake into the developing maize endosperm has been characterized using an in vitro system and appears to be driven by a gradient of hexose concentration between the pedicel and endosperm (Griffith et al., 1987a). Therefore, any process that involves sugar metabolism and compartmentation will influence assimilate partitioning and sink strength. It is obvious that enzymes involved in sugar transport and carbohydrate metabolism, in particular those that initiate sugar breakdown and those that are important in diverting sugar flux into storage compounds, are important in assimilate partitioning. In maize kernels, invertase is a good example of such an enzyme.

Porter et al. (1985) demonstrated that 74 to 90% of the total sugars within the pedicel parenchyma and placental-chalazal tissue (P-C) of wildtype maize are hexoses. Using pulse labeling techniques, it was demonstrated that labeled glucose and fructose predominate in the upper pedicel and lower portions of the endosperm (Shannon, 1968; 1972). Many other studies have shown the existence of sucrose inversion by invertase in maize kernel development (Doehlert and Felker, 1987; Cheng and Chourey, 1996). It has
been suggested that sucrose hydrolysis generates the driving force for assimilate partitioning into maize kernels by maintaining a sucrose gradient between the source and the apoplasm of the post-unloading zone and also the hexose gradients between the post-unloading apoplast zone and the endosperm tissue beyond the BETC (Shannon et al., 1993; Zamski, 1996). The fact that the development of invertase-defective kernels is seriously disrupted supports the putative importance of invertase in kernel development (Miller and Chourey, 1992). Because of the deficiency of invertase in the phloem post-unloading zone of the \textit{mn1} mutant, it is presumed that this mutant does not establish the sugar concentration gradients necessary for driving assimilate influx into its sink. It has been proposed that the invertase-mediated maintenance of photosynthate gradients between pedicel and endosperm constitutes a rate-limiting step in structural stability of maternal cells as well as normal development of the endosperm and kernel (Shannon et al., 1993). Previous study has suggested that the reduced amount of sugars and high ratio of sucrose to monomer sugars in \textit{mn1} kernels may lead to its low mass and withered phenotype at maturity (Shannon et al., 1993).

The mechanism of assimilate transport and partitioning into maize kernels is an unsolved problem that has intrigued people for decades (Shannon, 1968; 1972; Boyer and Shannon, 1986; Porter et al., 1986; Ho et al., 1991; Boyer 1996; Koch et al., 1996; Sturm, 1999). I intended to further explore the mechanism of assimilate transport and partitioning into maize kernels by using the \textit{mn1} mutant which is deficient in kernel invertase. My initial (assimilate partitioning study) investigations focus on the role of invertase in assimilate transport and partitioning to maize kernels by comparing the compartmentation of carbohydrates in kernel tissues between self-pollinated wildtype and \textit{mn1} plants. I conducted a follow up experiment comparing assimilate allocation between wildtype and \textit{mn1} phenotype kernels on the same maize ear to minimize the impact of individual plant variance on the measurement. I compared assimilate transport kinetics between kernels of wildtype and \textit{mn1} phenotype on a split-pollinated plant at 10 DAP to establish whether these kernels behave similarly when invertase activity is low in both genotypes. Finally, I used a modification of the empty seed technique of Porter et al.
(1995) with kernels of split-pollinated plants at 21 DAP to determine if the assimilate transport would be enhanced if the weak endosperm sink of \textit{mn1} genotype were replaced by large agar sink.

My hypotheses were 1) that \textit{mn1} kernels would accumulate more sucrose and less hexose in pedicel tissue and less of all these sugars in endosperm tissue compared to wildtype kernels as a result of its invertase deficiency; 2) that wildtype kernels would develop a strong hexose sugar gradient between maternal and filial tissue while \textit{mn1} kernels would develop weak hexose gradients; 3) that the heterozygous Mn1mn1mn1 endosperm kernel would provide assimilate transport results comparable to homozygous wildtype kernels. My hypothesis for the agar influx experiment was that replacing the endosperm of the \textit{mn1} genotype would not enhance assimilate transport into the kernel agar trap because the invertase deficiency would limit the development of a strong sucrose gradient from the pedicel.

### 3.2 Results and Discussion

The experiments with the self-pollinated homozygous plants were completed before the split-pollination technique was developed. Four kernel growth stages, 10, 13, 16, and 22 DAP, were selected to bridge the transition period from before to after the start of rapid kernel grain filling. In the split-pollination experiments, 10 and 21 DAP were chosen to represent kernel growth stages before and after it was thought invertase activity became important to the development of maize kernels. Since the \textit{wt} and \textit{mn} kernels shared a common assimilate source in split-pollinated plants, direct comparison of the assimilate partitioning between the two kernel genotypes was possible.

It is known that invertases start to function in endosperm tissue to facilitate assimilate transport to kernels around 12 DAP (Doehlert and Kelker, 1987; Cheng et al., 1996), therefore a split-pollinated plant at 10 DAP was chosen to study whether a
difference exists in the kinetics of assimilate transport between \textit{wt} and \textit{mn} kernels before invertase is likely to influence the process. By 21 DAP, the process and pattern of assimilate transport in maize kernels have been well established and ears are in the active linear phase of dry weight accumulation (Porter et al., 1985). Also, kernels at 21 DAP are an optimal size for agar influx treatment.

\subsection*{3.2.1 Biomass measurement}

Mature \textit{mn1} and wildtype plants are similar in leaf number and plant height and no other differences in plant phenotype were found between wildtype and \textit{mn1} plants grown in the same environment (data not presented). But \textit{mn1} kernels were much smaller than wildtype kernels at maturity. No difference in pericarp dry weight was observed between the two genotypes (data not shown), but dry weights of kernel pedicel, P-C, endosperm, and embryo tissue varied significantly (Figure 3–1). Pedicel biomass increased slowly but significantly, in both \textit{mn1} and wildtype kernels over the 12-day measurement period (inset Figure 3–1 Pedicel). Overall, pedicel biomass was significantly higher for the \textit{mn1} than the wildtype kernels (p=0.046). It may be that the lack of invertase in \textit{mn1} pedicel tissue encourages carbohydrate retention and additional biomass accumulation. The significant genotype by hour interaction occurred because pedicels from wildtype plants had lower biomass at sequential harvests from 2 to 12 hours while pedicels from \textit{mn1} increase in biomass (inset Figure 3–1 Pedicel). The nature of tissue sampling along with differences in endosperm sink strength between the two genotypes may account for the interaction. By necessity, the upper 1/3 of the ear was removed at the 2-hour collection and the lower 1/3 of the ear at the 12-hour collection. Since the developmental sequence of the ear spikelets is acropetal, those at the ear base are older, and probably develop greater endosperm sink strength than those at the ear tip. Because of their strong sink strength, less assimilate accumulates in the pedicels of wildtype kernels at the ear base than at the ear tip. In contrast, the lack of endosperm sink strength in \textit{mn1} kernels encourages storage of assimilates in pedicel
Figure 3–1: The dry weight biomass of pedicel, P-C, and endosperm tissues in wildtype and mn1 kernels from self-pollinated plants as influenced by genotype (G), DAP (D), and hour of sampling (H) from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
tissue throughout the day, contributing to their slight increase in biomass with sequential sampling throughout the day.

There is no clear difference in biomass of P-C tissue between the two genotypes (Figure 3–1 Chalazal). Chalazal tissue biomass appeared to increase over the sampling period, likely associated with the maturing of the overall kernel. Since mn1 kernels accumulate less overall biomass than wildtype kernels, their P-C tissues also acquire less biomass. Also, it may be that the excised chalazal tissue included a few cell layers of BETC tissue. It is likely that BETCs accumulate biomass rapidly from 16 to 22 DAP, so any contamination of P-C tissue with BETC cells could account for the apparent increase in P-C tissue biomass.

Endosperm biomass increases significantly over time in both genotypes (Figure 3–1 Endosperm). Dry weight of wildtype endosperms increased exponentially while that of mn1 only developed a slow linear increase during the 10 to 22 DAP period. This response difference between the two genotypes accounts for the significant genotype by day interaction shown in Figure 3–1. A nearly significant increase in endosperm biomass was observed with increasing hour of harvest (inset Figure 3–1 Endosperm). This observation is most likely due to ear position of the spikelets from which the endosperms were excised. Embryos were separated from endosperm tissue in both genotypes at 16 and 22 DAP. Embryos from wildtype kernels were significantly larger than those from mn1 at both 16 and 22 DAP (inset Figure 3–1 Endosperm). Therefore, both endosperm and embryo tissue contribute to the overall kernels dry weight difference between the two genotypes.

Biomass accumulation by kernels of split-pollinated plants is similar to that of endosperm tissue of self-pollinated plants (Figure 3–2). Dry weight of heterozygous wt kernels increased exponentially while that of mn only had slow linear increases during 6 to 21 DAP (Figure 3–2). In both cases, the dry weight biomass accumulated in mn1 kernels is about one fifth of that in wildtype phenotype kernels.
3.2.2 Carbohydrate compartmentation

Most sucrose arrives at the pedicel after long distance transport through the vascular system. In pedicel tissue, the sieve elements anastomose and become indistinguishable from vacuolated pedicel parenchyma cells (Shannon, 1972). The pedicel parenchyma cells are interconnected by plasmodesmata and surrounded by a mass of spongy parenchyma tissue (Shannon, 1972). Overlying the pedicel parenchyma is a compressed cell layer which eventually becomes the closing layer (Kiesselbach and Walker, 1952). This region is separated from the lower endosperm by a group of large cells, the placento-chalazal cells (P-C), which begin to degenerate around 7 DAP and are devoid of cell contents at 22 DAP (Shannon, 1972). Thus, during much of the kernel growth period, the tissue region immediately underlying the endosperm consists primarily of apoplasmod free space. Using microautoradiography, it was demonstrated that assimilates move through the pedicel parenchyma region via the symplast (Felker and Shannon, 1980). Since no plasmodesmatal or vascular connections exist between the pedicel and endosperm, sucrose may move throughout the pedicel via the symplast, but

\[ R^2 = 0.9872 \]

\[ R^2 = 0.9894 \]

**Figure 3–2**: Dry weight biomass of endosperms from split-pollinated plants during 6 to 21 DAP. Bars indicate ± standard deviation of the mean (when values exceed symbol size).
transport from the pedicel parenchyma cells into BETCs must occur through the apoplast of the pedicel and/or P-C tissues (Felker and Shannon, 1980).

Most sucrose is hydrolyzed while passing through layers of different tissues before reaching endosperm cells. There is high invertase activity in the pedicel free space and BETCs of wildtype kernels (Doehlert and Felker, 1987; Miller and Chourey, 1992). The invertases, both the soluble and cell-wall bound forms, hydrolyze sucrose into glucose and fructose irreversibly. Therefore, it is a mixture of sugars entering into the apoplast free space of chalazal tissue and subsequently into endosperm and embryo tissue through the BETC. However, in \textit{mn1} kernels, there is no invertase activity detected in pedicel, P-C, or BETC tissues (Miller and Chourey, 1992; Cheng et al., 1996). It is likely that this enzyme deficiency alters the sugar composition of the various kernel tissues in \textit{mn1} genotype in a way consistent with the concept that sugar transport is disrupted.

\subsection*{3.2.2.1 In pedicel tissue}

Pedicel total nonstructural carbohydrate (TNC) percentage averaged 56.1 and 52.7 in wildtype and \textit{mn1} genotypes respectively. It was similar between the two genotypes across all sampling periods (\textit{Figure 3–3}). A combined analysis of variance revealed a significant 3-way interaction among genotype, DAP and hour of sampling. These interactions appear to be associated with a fluctuation in the TNC percentage in the pedicels of the wildtype rather than the \textit{mn1} genotype. These fluctuations may be artifactual in that only two replicates were measured in wildtype compared to four replicates in \textit{mn1} genotypes and not due to biological causes. Hour of sampling had little effect on TNC percentage of \textit{mn1} pedicels over the 12-day sampling (\textit{Figure 3–3}), probably because the endosperm of this genotype did not develop into a strong sink to draw carbohydrates from the phloem unloading zone of the pedicel. In wildtype kernels, however, a strong endosperm sink appeared to cause pedicel TNC to fluctuate throughout
the day (Figure 3–3). TNC percentages tended to be higher in late afternoon (6 hr) samples than that in the later evening (12 hr) samples. The TNC data indicate that substantial amounts of carbohydrates are available in the pedicel tissue of both genotypes for transport into the endosperm.

The sugar components making up the TNC in pedicel tissue differed significantly between wildtype and \textit{mn1} homozygous maize kernels (Figure 3–4). In general, sucrose percentage tended to be higher, while glucose and fructose percentages were lower in \textit{mn1} compared to wildtype pedicel tissue. In addition, sucrose percentage tended to increase with DAP while glucose and fructose percentage declined. It appears that the lack of invertase activity in \textit{mn1} caused sucrose to accumulate in the pedicel over time while glucose and fructose declined, presumable because of its translocation to the endosperm and its lack of formation by rapid hydrolysis of sucrose.
Figure 3–4: The percentage of carbohydrates (dry mass basis) in kernel pedicel tissues of homozygous wildtype and \textit{mn1} plants as influenced by genotype (G), DAP (D), and hour of sampling (H) after exposing the second leaf above the ear to a pulse of 14CO\textsubscript{2} in mid-morning from 10 to 22 DAP. Statistical parameters for sucrose and fructose are from separate ANOV analysis for each genotype because a combined analysis revealed a significant GxDxH interaction.
At the same time, starch percentage increases slowly, but significantly, with DAP in both genotypes (Figure 3–5).

3.2.2.2 In P-C tissue

The sugar composition of chalazal tissue was dramatically different in the mn1 compared to the wildtype genotype (Figure 3–6). Sucrose percentage was 6 times higher while glucose and fructose percentages were three times lower in mn1 compared to wildtype kernels. Sucrose percentage in chalazal tissue increased slowly in both genotypes with hour of day (inset Figure 3–6 Sucrose). Small fluctuations in glucose percentage also occurred with time of day, but no clear pattern over days was established (inset Figure 3–6 Glucose). No starch was detected in samples of chalazal tissue in a preliminary experiment, so starch analysis was not performed on chalazal tissues in this sugar compartmentation study.

Figure 3–5: The percentage of starch (dry mass basis) in kernel pedicel tissues of homozygous wildtype and mn1 plants as influenced by genotype (G) and DAP (D) after exposing the second leaf above the ear to a pulse of 14CO2 in mid-morning from 10 to 22 DAP.
Figure 3–6: Carbohydrate components as a percentage of tissue biomass in placental-chalazal tissue of homozygous wildtype and mn1 plants as influenced by genotype (G), DAP (D), and hour of sampling (H) after exposing the second leaf above the ear to a pulse of 14CO₂ in mid-morning from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
3.2.2.3 In endosperm tissue

Sugar composition of endosperm tissue also differed significantly between wildtype and *mn1* genotypes (*Figure 3–7*). Sucrose percentage was consistently 4 to 5 points higher in *mn1* compared to wildtype at all sampling dates (*Figure 3–7 Sucrose*). Sucrose percentages ranged from 10 to 15% at 10 DAP and declined to 3 to 6% at 22 DAP. Endosperm glucose and fructose percentages followed a similar pattern with DAP in the two genotypes (*Figure 3–7 Glucose, Fructose*). Values were highest, ranging from 6 to 12% at 10 DAP and declined rapidly to around 1% at 22 DAP. Wildtype endosperm had significantly higher hexose percentage than *mn1* at 10 DAP, but the differences disappeared in older kernels, causing a significant sugar by DAP interaction for both glucose and fructose. A combined analysis of variance for endosperm glucose percentage revealed a significant (p=0.002) genotype by day by hour of sampling interaction. When each genotype was analyzed separately, I found endosperm glucose percentage of both genotypes differed significantly with DAP, but it was not affected by hour of sampling or day by hour interaction in either genotype. The starch percentage in endosperm tissue was near 0% at 10 DAP, but increased significantly to near 60% at 22 DAP for both genotypes (*Figure 3–8*). No significantly differences were observed between genotypes or with hour of day. The dynamics of sugar composition in endosperm of wildtype and *mn1* genotypes are consistent with cells transitioning from newly formed to rapidly growing storage tissues. Starch percentages increase rapidly while hexose sugars, the compounds supporting starch formation, start the growth period at relatively high percentages and declined as starch accumulates. The consistently higher endosperm sucrose percentage observed in *mn1* compared to wildtype genotypes may be associated with reduced activity of enzymes in the starch synthesis pathway. The fact that the starch percentage in endosperm tissue was not different between the wildtype and *mn1* genotypes with DAP, even though biomass accumulation was much higher in wildtype compared to *mn1*, indicates that rate of starch synthesis during this time period is linked to existing sink size rather than limiting the formation of sink size.
Figure 3–7: Carbohydrate components as a percentage of kernel biomass in endosperm tissue of homozygous wildtype and $mn_1$ plants as influenced by genotype (G), DAP (D), and hour of sampling (H) after exposing the second leaf above the ear to a pulse of 14CO$_2$ in mid-morning from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
3.2.2.4 Ratios among sugars in tissues

The ratio of sucrose percentage to hexose percentage in kernel tissue of the two genotypes highlights the effect of the invertase deficiency in \textit{mn1} on tissue carbohydrate composition (Table 3–1). The ratios are calculated from sugar percentages within each tissue averaged over sampling hour for each treatment. The ratios of sucrose to hexose sugar in the wildtype genotype were lower than those in the \textit{mn1} genotype in all tissues and at all ages measured. The ratio was significantly lower in P-C tissue of wildtype compared to \textit{mn1} kernels in all kernel developmental stages. The sucrose to hexose ratio increased with age in all tissues of both wildtype and \textit{mn1} plants, but the increase was more dramatic in \textit{mn1}. These data show that the action of invertase in the post phloem unloading zone maintains high hexose sugar levels in wildtype kernels, presumably to maintain the sugar gradients needed for assimilate transport needed to support rapid kernel growth.

\textit{Figure 3–8:} Starch as a percentage of kernel biomass in endosperm tissue of homozygous wildtype and \textit{mn1} plants as influenced by genotype (G) and DAP (D) after exposing the second leaf above the ear to a pulse of 14CO\textsubscript{2} in mid-morning from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
If assimilates transported into maize kernels follow an apoplast route through the P-C tissue and strong sugar concentration gradients drive the transport process, these gradients should be measurable. I compared the sugar percentages measured in chalazal tissue with that in endosperm tissue from 10 to 22 DAP (Figure 3–9). The ratio of sucrose, glucose, and fructose in P-C to that in endosperm tissue differed significantly between genotypes, although there was an interaction with DAP for glucose and fructose. The ratio of sucrose between the two kernel tissues was significantly higher in \textit{mn1} than wildtype. The ratio increased slowly, but significantly, with time of day (hour) in both genotypes (inset Figure 3–9 Sucrose). In contrast to sucrose, the ratios of glucose and fructose between the two tissue types was significantly higher overall in wildtype than \textit{mn1} genotypes (Figure 3–9). For both hexose sugars, the ratio was near unity and the same for both genotypes at 10 DAP, but it rapidly increased to over 20 in wildtype kernels, but only to about 12 in \textit{mn1} kernels over the next 12 days. These data clearly establish that a much stronger hexose gradient developed between the P-C and endosperm tissue in wildtype compared to \textit{mn1} genotypes. In contrast, a stronger sucrose gradient developed between these tissues in \textit{mn1} compared to wildtype plants.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Days after Pollination} & \textbf{Kernel Tissue} & \textbf{Sucrose to Hexose Ratio} \\
& & \textbf{Pedicel} & \textbf{Pedicel} & \textbf{Endosperm} & \textbf{Endosperm} \\
& \textbf{wildtype} & \textbf{mn1} & \textbf{wildtype} & \textbf{mn1} & \textbf{wildtype} & \textbf{mn1} \\
\hline
10 & 0.45 ± 0.14 & 1.06 ± 0.04 & 0.03 ± 0.03 & 2.26 ± 0.99 & 0.44 ± 0.11 & 1.20 ± 0.08 \\
13 & 0.70 ± 0.06 & 1.62 ± 0.12 & <0.01 & 2.72 ± 0.36 & 1.52 ± 0.29 & 3.29 ± 0.33 \\
16 & 1.48 ± 0.42 & 1.93 ± 0.08 & 0.16 ± 0.03 & 3.02 ± 0.07 & 1.73 ± 0.33 & 5.56 ± 0.36 \\
22 & 1.71 ± 0.17 & 4.41 ± 0.18 & 0.28 ± 0.15 & 3.60 ± 0.78 & 2.70 ± 0.29 & 6.31 ± 0.33 \\
\hline
\end{tabular}
\caption{Ratios of sucrose percentage to hexose percentage in kernel tissues of the self-pollinated \textit{wt} and \textit{mn1} plants. The ratios were calculated from sugar percentage (dry mass basis) averaged over sampling hours from each treatment.}
\end{table}
3.2.3 Assimilate transport

A previous study on the distribution of $^{14}$C-labeled carbohydrates in immature maize kernels of wildtype maize genotype (Shannon, 1968) showed that a high proportion of carbon radioactivity was in hexoses within 6 hours after labeling the plant with $^{14}$CO$_2$. He suggested that sucrose was hydrolyzed to glucose and fructose prior to or during its movement from the terminal phloem elements in the pedicel and pericarp tissue into the storage cells of the endosperm. This suggestion was confirmed by later studies (Shannon, 1972; Felker and Shannon, 1980; Miller and Chourey, 1992). Hydrolyze of sucrose into hexoses by invertase at the maize kernel base appears to be crucial for normal kernel growth and development, and a deficiency in invertase activity leads to reduced sink strength in $mn1$ kernels (Miller and Chourey, 1992; Shannon et al., 1993; Cheng et al., 1996). Differences in assimilate transport between wildtype and $mn1$ plants provide supporting evidence for the influence of invertase in the regulation of assimilate transport and partitioning in maize kernels.

3.2.3.1 Tissue relative DPM

The dynamics of accumulation and loss of radioactive label over time by maize kernel tissues after exposing leaves to $^{14}$CO$_2$ reveals the kinetics of assimilate transport and may provide clues about how the transport process is regulated. Data of absolute radioactivity in plant tissues and metabolites are often confounded by high plant to plant variability. One way to minimize the effect of this variance is to normalize measured radioactivity in a specific tissue or metabolite by expressing the data as a percentage of the total radioactivity in the sampled organ. Averaged over sampling hour at 10 DAP, both wildtype and $mn1$ genotypes had nearly 60% of their kernel radioactivity (relative DPM) in pedicel tissue compared to only 12% in P-C and 30% in endosperm tissue (Figure 3–10). Pedicel tissue from $mn1$ retained approximately 60% relative DPM at
Figure 3–9: The ratios of sugar percentage in chalazal to that in endosperm tissue of homozygous wildtype and mn1 plants as influenced by genotype (G), DAP (D), and hour of sampling (H) after exposing the second leaf above the ear to a pulse of 14CO2 in mid-morning from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
each of the three remaining sampling dates while relative DPM in the pedicel tissue of wildtype kernels declined to 38, 15, and 22% at 13, 16, and 22 DAP, respectively. At the same time, the relative DPM of endosperm plus embryo tissue in wildtype kernels increased to 50, 80, and 65%, respectively, over the same time period while that in \textit{mn1} kernels remained nearly constant at 30% (Figure 3–10). Pedicel tissue relative DPM declined with hour of sampling after the mid-morning exposure of the maize plant to $^{14}$CO$_2$ while it increased with DAP in endosperm plus embryo tissue (Figure 3–10). Genotype did not significantly affect the relative DPM of P-C tissue, although significant genotype by DAP, genotype by hour, and genotype by DAP by hour interactions were observed (Figure 3–10).

In wildtype P-C tissue, the interactions were due primarily to fluctuations in relative DPM over the 12 day experimental period while in \textit{mn1} relative DPM at 2 hours declined with increasing DAP but it tended to increase over the same DAP period at 6 hours after $^{14}$CO$_2$ labeling (Figure 3–11). The decline in relative DPM in pedicel tissue of the wildtype genotype with DAP is consistent with the relationship between increasing sink strength and assimilate transport kinetics. As kernel sink strength becomes stronger between 10 and 22 DAP, $^{14}$C-assimilates are drawn to the kernel more rapidly following leaf exposure to $^{14}$CO$_2$. Consequently, average pedicel relative DPM declines with DAP and with hour of measurement (inset Figure 3–10 Pedicel). The relative DPM of P-C tissue shows the same response, although the trend is less clear. The lack of invertase activity in \textit{mn1} (Doehlert and Felker, 1987; Miller and Chourey, 1992) seems to alter this response such that little change occurs in pedicel or P-C tissue relative DPM over the time course of the experiment (Figure 3–10). In contrast, as kernel sink strength increases, relative DPM in endosperm plus embryo tissue increases with DAP in the wildtype, but not in the \textit{mn1} genotype (Figure 3–10).
Figure 3–10: Relative radioactivity in tissues of homozygous wildtype and *mn1* plants as a percentage of total kernel radioactivity (DPM) as influenced by genotype (G), DAP (D), and hour of sampling (H) after exposing the second leaf above the ear to a pulse of 14CO2 in mid-morning from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
3.2.3.2 Sugar relative DPM within tissues

The radioactive label in pedicel tissue carbohydrate accumulated primarily in sucrose (about 50%) in both genotypes (Figure 3–12). In the wildtype genotype most of the remaining label was in glucose (21%) and fructose (21%) while only 11% was in starch (Figure 3–13). In contrast, most of the radioactive label in the mn1 genotype pedicels that was not in sucrose was in starch (38%) and only small amounts were in...
glucose (5%) and fructose (3%). Clearly, the presence or absence of invertase activity in the pedicel significantly altered the dynamics of tissue carbohydrate metabolism. Significant interactions were observed in pedicel carbohydrate relative DPM for genotype by hour and DAP by hour factors (Figure 3–12), but these interactions did not alter the interpretations of overall radioactive label dynamics.

In general, the relative DPM of glucose, fructose and starch declined with hour of sampling in wildtype kernel pedicels, while it tended to be higher at the 6 hour compared to the 2 and 12 hour measurement time in the \textit{mn1} genotype. Starch relative DPM tended to decline with increasing hours after labeling in pedicels of \textit{mn1}, while the trend was unclear for the wildtype genotype (Figure 3–13). The change in pedicel starch relative DPM with hour after labeling varied with DAP (Figure 3–13). Differences over hour of sampling were small at 10 and 13 DAP, but at 16 and 22 DAP starch relative DPM was much higher at the 2-hour compared to the 6- and 12-hour measurement. These observations are consistent with the impact that increasing sink strength with DAP would have on carbohydrate flux through pedicel tissue (Shannon et al., 1993; Cheng and Choureay, 1999).

The distribution of radioactive label among carbohydrate components in P-C tissue was similar to that observed for pedicel tissue except that no significant changer occurred with DAP for any of these components (Figure 3–14). In \textit{mn1}, most of the radioactive label was in sucrose (about 80% relative DPM) while glucose and fructose accumulated only 12% and 8% of the label, respectively. Just the opposite occurred in P-C tissue of wildtype kernels. Sucrose contained only 22% of the radioactive label while glucose and fructose averaged 36% and 42%, respectively. Sucrose relative DPM tended to increase in both genotypes with hour of sampling while glucose and fructose relative DPM tended to decline in both genotypes (insets Figure 3–14). Significant genotype by hour interactions were observed for sucrose and fructose relative DPM (p value and data insets Figure 3–14), but these interactions do not alter the overall interpretation of the data.
Figure 3–12: Relative radioactivity of carbohydrates in pedicel tissues of homozygous wildtype and *mn1* plants as a percentage of total kernel radioactivity (DPM) as influenced by genotype (G), DAP (D), and hour of sampling (H) after exposing the second leaf above the ear to a pulse of 14CO2 in mid-morning from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
Most (68%, averaged over DAP and hour of sampling) of the radioactivity in endosperm carbohydrates of the *mn1* genotype was in sucrose compared to 37% for the wildtype genotype (*Figure 3–15*). The significant DAP by hour interaction observed for sucrose relative radioactivity in endosperm tissue was caused by a decline in relative DPM from 10 to 16 DAP at the 12-hour collection while it increased at the 2-hr and 6-hr collection periods (Sucrose DxH, *Figure 3–15*). During the same time period, the relative DPM of endosperm starch at the 12-hr sampling increased (*Figure 3–17*), indicating radioactivity endosperm sucrose was being rapidly converted to starch.

*Figure 3–13*: Relative radioactivity of starch in pedicel tissue as influenced by genotype and DAP at 2, 6, and 12 hours after exposing the second leaf above the ear to a pulse of 14CO₂ in mid-morning from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
In contrast, glucose and fructose relative DPM was significantly higher in endosperm tissue of wildtype (24% and 23%, respectively) than \textit{mn1} (11% and 7% respectively) kernels (Figure 3–16). Embryo carbohydrate relative radioactivity followed patterns similar to that observed for endosperm tissue of the two genotypes (p value insets, Figure 3–16). Significant interactions were observed for endosperm and embryo sucrose, glucose, and fructose relative radioactivity between genotype and day or genotype and hour (Figure 3–15, Figure 3–16). These interactions were caused primarily by differences in magnitude of the measured responses and not in their direction, so the overall interpretation of the data was not altered by the interactions.

The relative radioactivity of endosperm starch increased with DAP in a similar pattern for both genotypes (Figure 3–17). Only a small amount of the radioactive label accumulated in endosperm starch at the 2-hr and 6-hr sampling time (5 to 10%), but massive amounts of relative radioactivity were measured in endosperm starch in the 12-hr sample (Figure 3–17). No differences were observed between genotypes, but a significant day by hour interaction was observed over both genotypes. The interaction between DAP and hour of sampling occurred for starch because of the decline in starch relative radioactivity in the 12-hr sample between 16 and 22 DAP while it increased slightly for the 2-hr and 6-hr sampling (starch DxH, Figure 3–17). Although the \textit{mn1} genotype accumulated less endosperm biomass than wildtype, the percentage starch in that biomass and the dynamics of radioactive label accumulation by endosperm starch were similar for the two genotypes (Figure 3–17). It appears as if the movement of carbohydrate into the endosperm of both genotypes was adjusted to match the ability of that sink to utilize the assimilate.
Figure 3–14: Relative radioactivity of carbohydrates in P-C tissues of homozygous wildtype and \textit{mn1} plants as a percentage of total kernel radioactivity (DPM) as influenced by genotype (G), DAP (D), and hour of sampling (H) after exposing the second leaf above the ear to a pulse of 14CO\(_2\) in mid-morning from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
Figure 3–15: Relative radioactivity of sucrose (sucrose DPM as a percentage of tissue total carbohydrate DPM) in endosperm (En) and embryo (Em) tissue of homozygous wildtype and mn1 plants as influenced by genotype (G), DAP (D), and hour of sampling (H) after exposing the second leaf above the ear to a pulse of 14CO2 in mid-morning from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
Figure 3–16: Relative radioactivity of hexoses (hexose DPM as a percentage of tissue total carbohydrate DPM) in endosperm (En) and embryo (Em) tissue of homozygous wildtype and \textit{mn1} plants as influenced by genotype (G), DAP (D), and hour of sampling (H) after exposing the second leaf above the ear to a pulse of 14CO\textsubscript{2} in mid-morning from 10 to 22 DAP. The statistical probabilities were obtained from SAS GLM analysis of variance.
3.2.3.3 Comparison of kernel relative radioactivity in self- and split-pollinated plant

In split-pollinated plants, both wt and mn1 kernels share a common assimilate source and translocation path to the ear. The amount of radioactive assimilate transported to kernels of the two genotypes should be determined by the individual sink strength of each kernel genotype. I measured tissue relative radioactivity (tissue DPM/kernel DPM) of wt and mn1 kernels on a split-pollinated ear of one mn1 maternal plant over a 6-hour period.
period after exposing plant leaves to $^{14}$CO$_2$ at 10 DAP. Almost half of the kernel radioactivity was in pedicel tissue of both genotypes (Table 3–2). Endosperm tissue contained 31 to 38% relative kernel radioactivity while the P-C tissue contained 13 to 18%. These percentages were similar for both kernel genotypes and were comparable with that measured for kernels on homozygous wildtype and $mn1$ plants (Table 3–2). These data provide evidence that results obtained with experiments using homozygous wildtype and $mn1$ kernel genotypes are comparable with that obtained with split-pollinated $mn1$ plants.

Table 3–2: Tissue relative radioactivity (tissue DPM as a percentage of total kernel DPM) and rate of radioactivity change in different tissues over 6 hours after exposing leaves of both self- and split-pollinated maize plants to $^{14}$CO$_2$ at 10 DAP. The plants were grown in the greenhouse at different times.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Relative DPM (%)</th>
<th>Tissue Δ DPM/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>mn</td>
</tr>
<tr>
<td></td>
<td>Self</td>
<td>Split</td>
</tr>
<tr>
<td>Pedicel</td>
<td>58.2</td>
<td>42.8</td>
</tr>
<tr>
<td>P-C tissue</td>
<td>12.9</td>
<td>18.6</td>
</tr>
<tr>
<td>Endosperm</td>
<td>28.8</td>
<td>38.5</td>
</tr>
</tbody>
</table>

The accumulation of radioactivity in individual kernels reflects the net effect of import, export, utilization, and storage processes. Within the maize kernel, the normal path of assimilate flow is from the pedicel vascular system to the pedicel apoplast, P-C tissue, and endosperm or embryo. The rate of radioactive assimilate (DPM) accumulation by endosperm of $wt$ kernels appears to be double that of $mn1$ kernels between 3 and 6 hours after plant $^{14}$C labeling (Table 3–2). At the same time, the pedicel tissue of $mn1$ kernels appears to retain more and the P-C tissue less radioactivity than comparable tissues of $wt$ kernels. These data are consistent with the observation that there is a slower overall assimilate transport rate into $mn1$ than into wildtype kernels.
3.2.4 Agar influx study

Assimilate unloading processes are difficult to understand because unloading mechanisms apparently differ among crop species and among the various sink tissues within a plant (Patrick, 1991). The empty-seed technique has proved to be an effective way to study the unloading process in developing seeds and possibly in whole-plant assimilate partitioning and the associated regulation (Thorne and Rainbird, 1983; Porter et al., 1985; 1986; Patrick, 1991). The empty-seed technique takes advantage of the symplastic discontinuity between the maternal and filial tissue by replacing the developing seed with agar or osmotic solutions that accept assimilate from the maternal apoplast. It is a technique that allows the study of the physiological role of maternal tissue and of factors controlling sink strength (Porter et al., 1987). The maize kernel cup technique, a modification of the empty seed technique, has been used to study assimilate unloading from the maize pedicel. Through its use, Porter et al. (1985) observed that sucrose moves passively from the maize pedicel into the apoplast, where it is hydrolyzed to hexoses by invertase (Porter et al., 1985). The pedicel cups consist of pericarp, vascular and pedicel parenchyma tissues of kernels attached to the cob rachis so that assimilates from the source regions of the plant continue to enter the pedicel and move into the cup solution or agar trap at rates comparable to assimilate movement into endosperm of unmodified kernels (Porter et al., 1985). The kernel-cup technique has been demonstrated to be a reliable and repeatable technique in maize (Porter et al., 1985; 1986; 1987a; 1987b).

The accumulation of assimilate radioactivity in intact kernel endosperm was compared with that in kernel cup agar traps of \textit{wt} and \textit{mn1} kernels on split-pollinated plants (Figure 3–18). The pattern of radioactive assimilate transport into agar traps in kernel cups was similar to that of radioactivity accumulation in endosperm tissue of intact kernels within each genotype. Intact kernel endosperm and kernel cup agar traps of the \textit{wt} genotype accumulated significantly more radioactivity than comparable treatments of the \textit{mn1} genotype. In another split-pollination treatment, PCMBS, a known inhibitor of
invertase, was added to the agar solution for the \textit{wt} kernels. This treatment makes these kernels physiologically similar to \textit{mn1} kernels with respect to invertase activity. The radioactivity accumulation in \textit{wt} agar traps with PCMBS was similar to that observed for \textit{mn1} endosperm and \textit{mn1} agar traps (Figure 3–19). These results provide additional evidence that lack of invertase activity in maize pedicel, P-C and basal endosperm tissues of \textit{mn1} kernels contributes to their reduced growth.

\begin{center}
\begin{tikzpicture}
  \begin{axis}[
    title={Figure 3–18: Radioactivity in assimilate transported to intact endosperm tissue or agar traps in \textit{wt} and \textit{mn1} kernels of split-pollinated plants after exposing leaves a pulse of $^{14}$CO$_2$ at 21 DAP.},
    xlabel={Min after pulse labeling},
    ylabel={$10^3$ DPM/3 units},
    xmin=120, xmax=260,
    ymin=0, ymax=20,
    legend style={at={(0.5,0.5)},anchor=west,legend columns=2},
  ]
    \addlegendentry{Agar-wt}
    \addlegendentry{Agar-mn}
    \addlegendentry{En-wt}
    \addlegendentry{En-mn}
    \addplot coordinates {
      (120,2) (140,5) (160,10) (180,15) (200,20)
    };
    \addplot coordinates {
      (120,2) (140,5) (160,10) (180,15) (200,20)
    };
    \addplot coordinates {
      (120,2) (140,5) (160,10) (180,15) (200,20)
    };
    \addplot coordinates {
      (120,2) (140,5) (160,10) (180,15) (200,20)
    };
  \end{axis}
\end{tikzpicture}
\end{center}

Collectively, there are significant differences between the wildtype and \textit{mn1} kernels in the patterns of assimilate transport and partitioning into kernels from source tissue. The disrupted kernel development and growth in \textit{mn1} kernels appears to be the consequence of reduced assimilate import due to the inefficient driving force for assimilate flux and reduced sink strength. Invertase, which is deficient in \textit{mn1} kernels, seems to play an important role in creating and maintaining the driving force for assimilate flux into maize kernels and in regulating kernel sink strength.
Figure 3–19: The pattern of assimilate transport to intact endosperm (En) and agar traps (Agar) of wt and mn1 kernels of a split-pollinated plants after exposing leaves a pulse of $^{14}$CO$_2$ at 21 DAP. The agar traps of wt kernels were treated with PCMBs.
4.1 Introduction

Maize (Zea mays L.) is one of the most important crops in the United States and the world. Maize grain is used as animal feed and as a source of refined starch, oil and other food ingredients. Over the years, many studies have been conducted on the anatomy, biochemistry and genetics of maize, but few have addressed the mechanism and regulation of assimilate partitioning in the kernel during its development and growth. Because of its economic importance, maize kernel metabolism and especially the regulation of storage product accumulation deserve more attention. The maize kernel represents a well-defined system for analyzing caryopsis post-phloem assimilate transport and sink metabolism. Characteristics of the miniature1 (mn1) maize mutant make it useful in studies of assimilate partitioning in the maize kernel. The phenotype of mn1 maize plants is indistinguishable from that of wildtype plants. In contrast, mn1 kernels are much smaller in size and have a papery, top-dented pericarp. These kernels cease growth around 12 to 13 days after pollination (DAP) and at maturity weigh about one fifth that of the wildtype kernels (Miller and Chourey, 1992).

The maize mutant, mn1, was first described as a seed specific mutation by Lowe and Nelson (1946). Subsequently, Miller and Chourey (1992) characterized mn1 maize as defective in cell-wall bound invertase in basal endosperm and pedicel tissue. This group later showed that mn1 is a null mutation of the wildtype gene Mn1 that encodes a cell-wall bound invertase isozyme (Cheng et al., 1996). It is of great interest to determine
how the absence of one enzyme, invertase, impacts both the development of filial \textit{mn1} kernels and the subtending pedicel and placento-chalazal (P-C) maternal tissues. Invertase (\(\beta\)-fructofuranoside fructohydrolase, EC 3.2.1.26) is widely distributed in maize plants, occurring in roots, leaves, silks, kernels and some other tissues. There are two forms of invertase, soluble and cell wall-bound. The soluble form is localized predominantly in vacuoles and cytoplasm and the cell wall-bound form is ionically bound to the cell wall (Jaynes and Nelsons, 1971; Sturm, 1999). Each of the two forms of invertase is known to have several isozymes. No matter the isoform or the location, the function of invertase is to hydrolyze sucrose into glucose and fructose. In wildtype maize kernels, cell wall-bound invertase is located in basal endosperm and adjoining pedicel tissue (Doehlert and Felker, 1987). Its presence has been detected as early as 7 DAP by histochemical staining. The activity of invertase, both soluble and cell wall-bound forms, has been measured during maize kernel development (Shannon and Dougherty, 1972; Cheng et al., 1996). Cell wall-bound invertase activity is significantly higher than the soluble form, accounting for 90\% of the total kernel invertase activity from 8 to 32 DAP (Cheng et al., 1996). Activities of both enzymes peaked at 12 DAP. On the other hand, \textit{mn1} kernels have almost undetectable levels of invertase activity at all developmental stages measured (Cheng et al., 1996). Invertase plays important roles physiologically in sugar allocation and partitioning between source and sink regions of plants (Roitsch, et al., 1995; Tang et al., 1999). In general, invertase localized in vacuoles is believed to be associated with the regulation of hexose levels in certain tissues and in the use of sucrose stored in vacuoles, while cell wall-bound invertase is associated with the rapidly growing tissues and plays important roles in phloem unloading and source/sink regulation (Roitsch and Tanner, 1996; Sturm, 1999). However, the mechanism through which a deficiency of invertase in \textit{mn1} kernels causes its phenotype is still unknown.

The maize endosperm is a product of double fertilization. The sperm fuses with the central cell and the fertilized central cell develops into endosperm. Since the central cell has two nuclei with each having one copy of the haploid genome, as does the sperm
nucleus, endosperm nuclei are triploid; each nucleus contains three copies (3C) of genetic material (where C represents the DNA content of the haploid genome). Another product of double fertilization is the embryo which is 2C. The embryo and endosperm cells are surrounded initially by nucellar tissue and pericarp, both of which are maternal tissues. The nucellar tissue is the main nutrient source for the developing endosperm and embryo in their very early growth stages. As the nutrients in nucellar tissue are consumed and the nucellar tissue degenerates, phloem transported assimilates become a vital energy source for kernel growth. Kernel development is closely connected with assimilate transport processes and endosperm metabolism. Kernels are strong sinks that import assimilates from the phloem. Sucrose, a principal product of photoassimilation in maize, is the major form in which carbohydrate is translocated from leaves to the rest of the plant for growth and accumulation of storage reserves (Ap Rees, 1987). Sucrose not only functions as a transport metabolite, but also it contributes to the osmotic driving force for phloem translocation (mass flow) and serves as a signal to regulate specific genes in a variety of different tissues (Koch, 1996). Physiological and molecular studies show that sucrose transport is highly regulated at multiple levels of biological organization and in response to changing sucrose concentrations (Lalonde et al., 1999). Sink organs (e.g. maize kernels) have different capacities (referred to as sink strength) to attract sucrose transported from source leaves, which influences assimilate partitioning to the grain. The molecular nature of this process has been identified cleavage of sucrose by cell wall invertase at the site of phloem unloading as one of the key steps (Weber et al., 1996; Weber et al., 1997; Sturm and Tang 1999). Because of the function of invertase and its critical location in kernels, suggestions have been raised that invertase mediates a concentration gradient of sugars between pedicel and endosperm tissue which contributes to the driving force for assimilate partitioning into maize kernels for their normal growth (Shannon et al., 1993). Studies on assimilate partitioning have been conducted in wildtype maize (Shannon, 1968; 1972) and other cereals (Darussalam et al., 1998), but no quantitative information is available so far on either mn1 kernel development or assimilate partitioning into mn1 kernels.
Plant hormones play important roles in regulating division, differentiation, and cell growth. Photoassimilate distribution in plants is predominantly under sink control and sink-produced plant hormones appear to play an important role in this process (Wardlaw, 1990). Cytokinins are involved in the control of numerous and important processes associated with plant growth and development (Chen, 1998). The auxin IAA stimulates photoassimilate flow to and within developing wheat grains by a primary action on the membrane exchange of solutes to and from the endosperm cavity and leads to more starch biosynthesis by increasing supplies of sucrose to the endosperm (Darussalam et al., 1998). The levels of ABA in plant cells usually change when physiological and environmental stress occur (Mambelli and Setter, 1998; Ahmadi and Baker, 1999). In maize kernels, auxin, cytokinin and ABA have been associated with abnormal kernel growth and abortion under heat and water deficit stress (Jones et al., 1990; Ober et al., 1991; Lur and Setter, 1993a; 1993b; Cheikh and Jones, 1994). It is reasonable to anticipate that there may be significant differences in the quantity of hormones between \textit{mn1} and wildtype kernels.

A comparative study of sink strength, hormone levels, and assimilate partitioning between \textit{mn1} and wildtype maize kernels will help advance our understanding of the involvement of assimilate transport in maize kernel growth and development. However, it is difficult to compare the growth and development of kernels growing on different individual plants because of inherent plant-to-plant variation. In order to minimize the impact of plant to plant variability, I used a split-pollination technique in which half the silks on a homozygous \textit{mn1} plant were pollinated with wildtype pollen and the other half were self-pollinated. As a result, kernels on one side of the split-pollinated ear are homozygous (genotypes of the endosperm and embryo are \textit{mn1mn1mn1} and \textit{mn1mn1}, respectively) and those on the other side of the ear heterozygous (genotypes of the endosperm and embryo are \textit{Mn1mn1mn1} and \textit{Mn1mn1}, respectively). The single copy of \textit{Mn1} in the endosperm is sufficient to restore the kernels to full size, like the homozygous wildtype phenotype. All the results presented in this report are from split-pollinated plants. For convenience, we call the heterozygous kernels with the wildtype phenotype
wt kernels and the homozygous kernels with the mn1 phenotype mn kernels throughout this study. Both wt and mn kernels of split-pollinated ears were harvested at the same time and various cytological and metabolite measurements were made. Reported here are the results of a comparative study of wt and mn kernels to identify factors that regulate and control assimilate partitioning during kernel growth and development.

4.2 Results and Discussion

4.2.1 Biomass accumulation

The split-pollination procedure used makes it possible to compare the growth and development of the two kernel phenotypes produced by two genotypes on a single ear. Cheng et al. (1996) reported that heterozygous kernels whose endosperms only have one copy of the wildtype gene (Mn1mn1mn1) phenotypically resemble homozygous wildtype kernels in size and dry mass even though they only have about 21% of the total invertase activity of homozygous wildtype kernels. It is difficult to detect visually the difference between wt and mn genotypes from 4 to 8 DAP. Differences were detectable by eye after 9 DAP. The distal pericarp of wt kernels develops fully while that of mn kernels becomes dented, indicating an inhibition of growth of the enclosed endosperm (Figure 4–1). This phenotypic character was consistent in mn kernels to maturity.

Both whole-kernel and endosperm dry biomass of wt kernels increased exponentially from 6 to 12 DAP while that of mn kernels was nearly linear over the same growth period (Figure 4–2). Divergence in dry weight accumulation between wt and mn whole kernels and endosperm tissues started at 9 DAP and increased for the duration of the experiment.
Figure 4–1: A split-pollinated ear at 24 DAP. Homozygous mn1 kernels are on the upper half of the ear and kernels with Mn1mn1mn1 endosperm genotype displaying a full kernel phenotype are on the lower half of the ear.
The phenotypes of \textit{mn} and \textit{wt} kernels from split-pollinated ears resemble those of homozygous \textit{mn1} and wildtype parent kernels, respectively. But the success of using split-pollination in this study depends on whether it produces kernels that resemble homozygous kernels physiologically. Supporting evidence for this resemblance is the presence and absence of invertase activity in these kernels. Kernels harvested from split-pollinated ears of 4 to 16 DAP were used in histochemical staining of invertase (\textit{Figure 4–3}). Heterozygous \textit{wt} kernels (first and third row) showed strong invertase activity with intense black staining at the base of endosperm and top of pedicel as early as 4 DAP (first two kernels on the left in the first row). Staining density increased along with the kernel size from 4 to 11 DAP and remained high up to 16 DAP. This observation is consistent with a recent study on kernel cell wall bound invertase activity in wildtype maize which reveals that kernels exhibit invertase activity at 4 DAP and it increases dramatically with kernel development (Carlson and Chourey, 1999). Meanwhile there was no positive reaction in \textit{mn} kernels (second and forth row). The brown color seen at the base of endosperm of the \textit{mn} kernels is the same as that observed in \textit{wt} and \textit{mn} kernel controls in

\textit{Figure 4–2}: Biomass accumulation in kernels and endosperm tissue from split-pollinated plants grown in the greenhouse. Bars indicate ± standard deviation of the mean (where values exceed symbol dimensions).
which no sucrose was added as a substrate for invertase cleavage to hexoses. Thus, I conclude the brown color in the $mn$ kernels (Figure 4–3) is not due to invertase activity. This result, in combination with other invertase characteristics of $mn1$ and wildtype kernels reported in previous studies (Miller and Chourey, 1992; Cheng et al., 1996; Cheng and Chourey, 1999), provide evidence that kernels produced by split-pollination are valid for comparative studies of growth and development of homozygous $mn1$ and heterozygous wildtype kernels.

### 4.2.2 Sink development in $wt$ and $mn$ kernels

Visual observations and biomass data indicated that $mn$ kernel growth is severely inhibited after 12 DAP, resulting in a small final kernel size. Flow cytometry was used to estimate endosperm nuclei numbers between 6 and 21 DAP to assess kernel sink size. Since each maize endosperm cell has one nucleus, the nuclei number per endosperm also represents endosperm cell number per kernel. No difference was found in endosperm cell number per endosperm between $wt$ and $mn$ kernels at 6 DAP, the earliest time measured (Figure 4–4 A). Differences appeared after 6 DAP and were significant by 12 DAP. There was no detectable increase in endosperm cell number in either genotype after 12 DAP. Endosperm cell number in $mn$ kernels was about 50% of that in $wt$ kernels when both of them reached their highest values about 16 DAP. These observations are consistent with previous studies reporting that mitotic activity reaches a peak in the developing endosperm between 8 and 10 DAP and after 12 DAP endosperm cells mainly enlarge and differentiate rather than divide (Kowles and Phillips, 1988). The apparent decline in cell number after 16 DAP may be due to interference with the flow cytometry detection of nuclei caused by the large starch granules.

Kernel starch makes up about 70% of the mature kernel dry weight. Maize kernels store starch as granules in amyloplasts inside endosperm cells. The size of the starch granule enlarges as starch synthesis and accumulation proceed during kernel
Figure 4–3: Detection of invertase in kernels harvested from a split-pollinated ear showing that the full size *wt* kernels have invertase activity at basal endosperm and upper pedicel tissue, but *mn* kernels do not. The slices in the rows one and three are from *wt* kernels sampled at 4, 4, 6, 6, 8, 8, 9, 11, 11, 13, and 16 DAP; and the slices in the rows two and four are from *mn* kernels sampled at 6, 6, 7, 7, 9, 9, 11, 13, and 16 DAP (from left to right). The narrow, dark zone at the base of endosperm in the *mn* slices was also present in the sucrose depleted control slices from both the *wt* and *mn* kernels and thus was not considered to be due to invertase activity.
Figure 4–4: Endosperm cell number (A), starch granule number (B) and the starch granule number per endosperm cell (C) in kernels at various days after pollination of split-pollinated plants. Bars indicate ± standard deviation of the mean (where values exceed symbol dimensions).
growth. Starch granule numbers increased rapidly in endosperms of both \textit{wt} and \textit{mn} kernels between 9 and 12 DAP, but the rate of increase in \textit{mn} kernel was about half that observed in \textit{wt} kernels (\textit{Figure 4–4} B). Between 12 and 21 DAP, starch granule number per endosperm increased slowly and at similar rates for the two kernel types. Consequently, by 21 DAP \textit{mn} kernels accumulated about half the number of starch granules as \textit{wt} kernels because of the differences in the rate of granule formation from 9 to 12 DAP (\textit{Figure 4–4} B). When starch granule numbers are compared per endosperm cell, however, there was no difference between the two kernel genotypes (\textit{Figure 4–4} C). This indicates that most of the difference in kernel biomass accumulation between the two genotypes is related to their differences in endosperm cell number per kernel.

Differences were found not only quantitatively but also qualitatively in endosperm cells between the two kernel types. Maize endosperm cells undergo active nuclear DNA endoreduplication during early development, which leads to multiplied DNA content (Kowles and Phillips, 1985). Nuclear DNA endoreduplication profiles determined by flow cytometry differed between \textit{wt} and \textit{mn} kernels at 16 DAP (\textit{Figure 4–5}). Based on the assumption that each endosperm cell has one nucleus, the peaks in the profile represent the numbers of endosperm cells (each nucleus is one event) with a particular DNA content. At 16 DAP \textit{wt} kernels have more endosperm cells with double DNA content (6C) than \textit{mn} kernels.

Expressing endosperm cell number at each DNA content level as a percentage of the total endosperm cell number at each sampling date reveals the endosperm nuclei endoreduplication pattern in the two kernel genotypes (\textit{Figure 4–6}). Most of the cells contained the basic (3C) or double the basic (6C) DNA content. In \textit{wt} kernels, however, the percentage of endosperm cells with the basic DNA content of 3C declined faster and reached a lower percentage over the sampling period than in \textit{mn} kernels. The decline of the percentage of endosperm cells with 3C in \textit{wt} kernels was matched by an increase in the percentage of cells at 6C. There was no significant increase in 6C nuclei percentage
There were no significant differences in nuclei with 12C, 24C, 48C and 96C levels of DNA between the two kernel types (data not shown).

Figure 4–5: Histogram illustrating the number of endosperm nuclei in each DNA-content size class of each kernel genotype from a 16 DAP split-pollinated ear, as determined by flow cytometry. Fluorescence was plotted on a logarithmic x-axis. The numbers expressed on the y-axis are nuclei counts in proportion to the size classes. The peaks are DNA-content size classes 3C, 6C, 12C, 24C 48C, and 96C (from left to right). The upper histogram is from a \( wt \) kernel and lower one \( mn \) kernel.

in \( mn \) kernels. There were no significant difference in nuclei with 12C, 24C, 48 C and 96C levels of DNA between the two kernel types (data not shown).
4.2.3 Sink related enzyme activities

Maize endosperm comprises approximately 80% of the mature kernel biomass and it is composed of 88% starch. Therefore any metabolic factor involved in endosperm starch synthesis is likely to be important for kernel sink activity development and achievement of high final kernel weight. Starch is synthesized and accumulates in amyloplasts inside endosperm cells. The enzymes involved in maize endosperm starch synthesis are identified and well studied (Shannon et al., 1998). They are AGPase (EC 2.7.7.27), starch synthase (EC 2.4.1.21), starch branching enzymes (SBEs, EC 2.4.1.18) and starch-debranching enzymes (). Endosperm AGPase activity per kernel increased from 10 to 21 DAP in both kernel types, but the increase was much faster in wt compared
to \( mn \) kernels (Figure 4–7A). At 21 DAP, AGPase in \( mn \) kernels was only 20% of that in \( wt \) kernels (Figure 4–7 A). AGPase activity exhibited the same pattern and trend when expressed per cell and per amyloplast (data not shown). Sucrose synthase (Figure 4–7 B) and starch synthase (Figure 4–7 C) activities also differed significantly between \( mn \) and \( wt \) kernels. In both cases, \( wt \) kernels had significantly higher activity than their \( mn \) counterparts. A similar result was found in starch branching enzyme activity (data not shown). Conclusively, \( mn \) kernels have overall lower sink activity than \( wt \) kernels. It’s unclear why we observed lower sucrose synthase activity in \( mn \) kernels than in \( wt \) kernels while no difference of the sucrose synthase activity between the two genotypes was found in Miller and Chourey (1992).

### 4.2.4 Endosperm hormone differences between genotypes

Hormones play an important role in plant cell division and differentiation. Seldom does a single hormone operate to regulate a cell or tissue process. Rather, hormones function together in complex ways and the balance among hormones in plant cells is more critical in determining cell response than the amount of one single hormone (Lur and Setter, 1993b; Jensen and Bandurski, 1994; Coenen and Lomax, 1997). This complexity of hormone action and its influence on kernel growth and development has been observed in maize grown under water deficit and heat stresses (Ober et al., 1991; Cheikh and Jones, 1994; Artlip et al., 1995). Previous studies on plant hormones indicated that cytokinin, auxin and ABA levels are related to maize kernel growth (Ober et al., 1991; Lur and Setter, 1993a,b; Cheikh and Jones, 1994; Artlip et al., 1995), so we measured their content in endosperm tissue in kernels of both genotypes from split-pollinated ears to assess whether differences were linked to genotype.

ABA mediated modification of growth is a fundamental response of plants to adverse environmental cues. Since cell ABA levels normally increase as plant exposure
Figure 4–7: Activities of AGPase (A), sucrose synthase (B) and starch synthase (C) per endosperm of *wt* and *mn* kernels from split-pollinated ears from 8 to 21 DAP. Bars indicate ± standard deviation of the mean (where values exceed symbol dimensions).
to stresses occur, ABA is considered to be involved in a stress signal transduction system in cells (Myers et al., 1990; Cheikh and Jones, 1994). As the phenotype of *mn1* kernels appears similar to that of kernels under stresses, it was suspected that ABA level will be different between kernels of the two genotypes on split-pollinated ears. Endosperm ABA levels changed with kernel age on a per kernel (*Figure 4–8 A*) and a per gram fresh weight (*Figure 4–8 B*) basis. In each kernel genotype, endosperm ABA content per kernel steadily increased from 9 to 21 DAP (*Figure 4–8 A*), but the increase was more rapid in *wt* compared to *mn* kernels. When expressed on a mass basis, however, the differences in ABA concentration between the two kernel types were reversed. Endosperm tissue from *mn* kernels contained a higher concentration of ABA per gram fresh weight compared to *wt* kernels through the 9 to 21 DAP growth period (*Figure 4–8 B*).

A reversed pattern was observed for IAA levels in kernels of the two genotypes on split-pollinated plants (*Figure 4–9*). On a per endosperm basis, *wt* kernels contained significantly more IAA than *mn* kernels (*Figure 4–9 A*). IAA levels in *mn* endosperm remained low throughout the measured period of kernel development. Endosperm IAA concentration in both genotypes peaked early during kernel development (*Figure 4–9 B*). However, the peak of IAA concentration was higher and occurred a day earlier in *wt* kernels than in *mn* kernels. Endosperm IAA concentration level remained high in *wt* kernels throughout the 21-day growth period, while it declined in *mn* kernels after 12 DAP.

In maize, cytokinin accumulation has been shown to be associated with endosperm cell proliferation (Jones et al., 1990; Lur and Setter, 1993b; Dietrich et al., 1995). The observation that maximal kernel cytokinin accumulation precedes or occurs simultaneously with endosperm cell proliferation suggests that cytokinins may be a critical regulatory component of kernel development. There are different forms of cytokinin in higher plants (Chen, 1998). Zeatin and Zeatin Riboside (ZR) are the most
investigated active forms of cytokinins in maize kernels (Jones et al., 1990; Lur and Setter, 1993b; Dietrich et al., 1995). There were no differences in either zeatin or ZR concentration between \textit{mn} and \textit{wt} kernel endosperm (Figure 4–10) during early endosperm development (6 to 12 DAP). In both \textit{wt} and \textit{mn} kernels, zeatin and ZR content per gram fresh weight peaked at 10 and 8 DAP, respectively. Zeatin and ZR levels in \textit{wt} kernels declined to low levels during the rest of the measured period (12 to 18 DAP). Both zeatin and ZR reached another concentration peak around 15 DAP in \textit{mn} kernels while values in \textit{wt} kernels remained low. In contrast to ZR, the zeatin response

\textit{Figure 4–8:} ABA levels on per kernel (A) and per gram fresh weight (B) basis in kernels from split-pollinated ears from 9 to 21 DAP. Bars indicate ± standard deviation of the mean (where values exceed symbol dimensions).
was so specific that it only occurred at 15 DAP. I also measured another form of cytokinin, zeatin-9-glucose, but no differences were detected between the two kernel types (data not shown).

Collectively, hormones levels differ, at some growth stage greatly, between the two genotypes. Higher ABA levels as well as lower IAA levels may indicate that mn kernels were under stress (physiological stress) compared to wt kernels. Mixed information was got when cytokinin data were interpreted alone. In order to well understand factors regulating the endosperm cell growth between the two genotypes, the

*Figure 4–9:* IAA levels on per kernel (A) and per gram fresh weight (B) basis in kernels from split-pollinated ears from 9 to 21 DAP. Bars indicate ± standard deviation of the mean (where values exceed symbol dimensions).
ratios between cytokinins to ABA and IAA to cytokinins during 9 to 18 DAP were determined (Table 4–1). The wt kernels had higher cytokinins to ABA ratios than mn kernels during the early period (9 to 12 DAP) and the pattern reversed in the later period (13 to 18 DAP). The wt kernels had higher IAA to cytokinins ratio than mn kernels during the whole measured period (9 to 18 DAP).

Figure 4–10: Zeatin (A) and Zeatin Riboside (ZR, B) levels per gram fresh weight basis in kernels from split-pollinated ears from 6 to 18 DAP. Bars indicate ± standard deviation of the mean (where values exceed symbol dimensions).
The sources of assimilates for plant growth are the leaves during most maize developmental stages. Assimilates from leaves undergo phloem loading, phloem unloading and post-unloading (also called post-sieve element transport) in order to reach sinks from the source (Oparka, 1990; Patrick and Offler, 1995). Previous studies have demonstrated that maize kernel development is dependent on a supply of phloem-transported sucrose (Felker and Shannon, 1980; Porter et al., 1985). After phloem unloading and before reaching kernel tissue of the filial generation, sucrose traverses post-unloading tissue consisting of the distal part of the pedicel parenchyma, the placento-chalazal cells and the basal endosperm transfer cells (BETC) (Oparka, 1990). In wildtype kernels, acid invertase is present in this post-unloading zone and hydrolyzes sucrose into glucose and fructose as sucrose is unloaded into the tissue apoplastic space. Therefore, it is a sugar mixture that moves into developing kernels. Split-pollinated plants with kernels at 10 and 21 DAP representing early and late stages of maize kernel development, respectively, were selected to study the influence of invertase on sugar distribution in kernels.

Table 4–1: Ratios between Zeatin (Z) plus Zeatin Riboside (ZR) to ABA and between IAA to Z plus ZR in kernels from split-pollinated kernels harvested from 9 to 18 DAP.

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4.2.5 Sugar distribution in kernels from split-pollinated plants

The sources of assimilates for plant growth are the leaves during most maize developmental stages. Assimilates from leaves undergo phloem loading, phloem unloading and post-unloading (also called post-sieve element transport) in order to reach sinks from the source (Oparka, 1990; Patrick and Offler, 1995). Previous studies have demonstrated that maize kernel development is dependent on a supply of phloem-transported sucrose (Felker and Shannon, 1980; Porter et al., 1985). After phloem unloading and before reaching kernel tissue of the filial generation, sucrose traverses post-unloading tissue consisting of the distal part of the pedicel parenchyma, the placento-chalazal cells and the basal endosperm transfer cells (BETC) (Oparka, 1990). In wildtype kernels, acid invertase is present in this post-unloading zone and hydrolyzes sucrose into glucose and fructose as sucrose is unloaded into the tissue apoplastic space. Therefore, it is a sugar mixture that moves into developing kernels. Split-pollinated plants with kernels at 10 and 21 DAP representing early and late stages of maize kernel development, respectively, were selected to study the influence of invertase on sugar distribution in kernels.
The content and radioactivity of sugars in endosperms sampled at 3, 6 and 9 hours after the split-pollinated ears were labeled with $^{14}$CO$_2$ were determined. Sugar content differences between $mn$ and $wt$ kernels were determined by subtraction of sugar content in $wt$ from that in $mn$ endosperms (Figure 4–11). At 10 DAP, $mn$ and $wt$ kernels have similar sucrose content (Figure 4–11 A). At 21 DAP, $mn$ kernels have significantly higher sucrose content than $wt$ with $p$-value of 0.001. This shows that sucrose is the main form of sugar present in $mn$ endosperms and suggests that utilization of sucrose for starch synthesis in $mn$ endosperms is disrupted. Kernel glucose and fructose contents were much lower than sucrose in both kernel genotypes (Figure 4–11 B compared to A). Endosperm glucose content was similar between the two genotypes ($p$-value of 0.3), while fructose content was significantly higher in $wt$ than in $mn$ kernels ($p$-value of 0.03). These data suggest that the presence or absence of invertase activity already influences the sugar partitioning pattern in kernels at 10 DAP. Hydrolysis of sucrose by invertase in $wt$ kernels occurred at 10 DAP and was significant enough to cause differences in glucose and fructose content between $mn$ and $wt$ kernels, even though no difference in the total sucrose content was found between the two kernel genotypes. I suspect that the amount of sucrose hydrolyzed by invertase in $wt$ kernels was small compared to the sucrose pool present in the tissue, but large enough to influence the much smaller pool size of the fructose. Sucrose from phloem is undoubtedly used to synthesize starch by endosperm cells with or without hydrolysis and eventually stored as starch granules in endosperm cells. By 21 DAP, starch is the main form of carbohydrate in wildtype kernels, instead of sucrose or hexose (Shannon, 1972). The difference in kernel glucose and fructose content between the two genotypes was not significant (according to $p$-value $> 0.05$) at 21 DAP (Figure 4–11 B). My interpretation for this observation is that glucose and fructose are not the main form of carbohydrate storage at this kernel developmental stage, so pool sizes do not reflect the flux of assimilates through the tissue.

Previous work shows that shortly after maize plants are exposed to $^{14}$CO$_2$, most of the radioactivity in the kernels is present in the hexoses and later the proportion of $^{14}$C in sucrose increases while that in the hexoses declines (Shannon, 1968). A similar pattern
of $^{14}$C movement was observed in the kernels of split-pollinated ears collected at 3, 6 and 9 hours after $^{14}$CO$_2$ exposure. Analysis of variance of these data revealed no genotype by time interaction. Consequently, differences in specific activity of carbohydrate fractions between $mn$ and $wt$ kernels were averaged over the three time periods (Figure 4–11 C). At 10 DAP, there was no significant difference in specific activity of sucrose between the two genotypes, but $wt$ kernels had significantly higher glucose and fructose specific activity than $mn$ kernels ($p$-values of 0.06 and 0.003, respectively). At 21 DAP, the specific activity of sucrose, glucose and fructose was significantly higher in $wt$ than $mn$, $p$-value of 0.001. This indicates the $^{14}$C photosynthate flux into $wt$ kernels was faster than into $mn$ kernels at 21 DAP. A faster assimilate flux is usually associated with a higher mass flux of the labeled assimilate.
Figure 4–11: The difference (mn-wt) in sugar content and 14C specific activity of sugars in kernels from split-pollinated ears at 10 (solid bar) and 21 DAP (hollow bar). Values plotted are least square means determined by SAS Proc Mixed program. Bars indicate ± standard error of the mean.
Chapter 5
Discussion and Perspectives

The study of maize kernel development is of great important since kernel development is closely associated with yield. Although previous research has shown that physiological and environmental factors influence maize kernel development, in part by affecting assimilate metabolism in kernel tissue, there is no consensus among researchers on the mechanism regulating assimilate allocation and partitioning to the maize kernel (Lur and Setter, 1993a,b; Cheikh and Jones, 1994; Artlip et al., 1995). Speculation has been made that several processes, like passive efflux of sucrose from the phloem, kernel pedicel cell turgor, or sucrose carrier proteins may be involved. My comparison of homozygous mn1 kernels and heterozygous wt kernels with wildtype phenotype grown on the same ear supports the model proposed by Shannon et al. (1993) that the driving force for assimilate movement into normally developing maize kernels is the sucrose-gradient between the leaves (source) and the kernel pedicel apoplasm and the concentration gradient of hexoses between the pedicel apoplasm and starch accumulating endosperm cells. Both gradients are maintained by the activity of cell-wall bound invertase in pedicel tissue at the base of the endosperm. The failure to create and maintain these sugar gradients because of the absence of invertase in mn1 kernels appears responsible for their reduced biomass accumulation and miniature phenotype.

Endosperm is the major reserve storage tissue of maize kernels. Its storage capacity is primarily established during the first 12 days after pollination and fertilization. Endosperms develop through the processes of cell division, cell enlargement, organelle proliferation, and the metabolic activities in the cells. Final endosperm mass (sink strength) depends on endosperm cell number, starch granule number in endosperm cells and the activities of starch synthesis related enzymes in endosperm tissue. The
development of sink strength is a complex process which regulates and controls assimilate allocation and partitioning (Ho, 1988; Weber et al., 1997; Weber et al., 1998). Endosperm development is made possible by the availability and delivery of sugars from the maternal tissue, firstly from nucellar tissue in the first few days after fertilization and later from phloem transport as nutrients in nucellar tissue are depleted. Even though sucrose is capable of entering endosperm cells passively (Shannon, 1968; Porter et al., 1985) and intact without invertase hydrolysis (Schmalstig and Hitz, 1987), a “driving force” mechanism is needed to maintain the sufficiency and continuity of sugar supply as the endosperm develops. Assuming the pathway(s) for assimilate utilization (starch synthesis) is not defective, this driving force can be generated by sugar gradients between different tissues. Cell-wall bound invertase in the P-C tissue and basal endosperm tissue can generate both sucrose and hexoses concentration gradients by hydrolyzing sucrose to its monomer constituents. Because of its special localization, the cell wall-bound invertase in the basal endosperm region of the maize kernel is critical for endosperm development. The hydrolysis of sucrose by invertase in basal kernel tissue alters the entire sugar environment of this tissue. Cell wall-bound invertase breaks down sucrose into glucose and fructose and creates a sucrose concentration gradient from terminal phloem cells to the apoplastic space of the P-C tissue and basal endosperm. It also raises the glucose and fructose concentration in this zone, creating a glucose and fructose concentration gradient between the apoplastic of the P-C tissue and basal endosperm tissues and the symplasm of the metabolically active endosperm cells. The generation and maintenance of sugar concentration gradients between these different tissues provides a mechanism that enhances assimilate transport into maize kernels by passive diffusion or facilitated membrane transport.

A significant difference in the sugar environment was observed between the two kernel phenotypes on split-pollinated ears. The reason for studying kernels from 10 and 21 DAP plants is that they represent the growth periods before and after the impact of the invertase deficiency. At 10 DAP, invertase starts to function and begins to play a role in sugar partitioning to the developing kernels (Doehlert and Felker, 1987; Miller and
Chourey, 1992). No significant difference was found between wt and mn kernels in either sucrose content (A) or specific activity at 10 DAP (Figure 4-11 C). In contrast, the content (Figure 4-11 B) and specific activity (Figure 4-11 C) of glucose and fructose were significantly higher in wt than in mn kernels at 10 DAP, with p-value of 0.06 and 0.003, respectively. This shows that even by 10 DAP the sugar environment is already different between the two genotypes, presumably because of the presence of invertase in the wt kernels. In both genotypes, the concentration of sucrose in the endosperm is much higher than that of hexoses and a reduction in sucrose due to inversion or other metabolism was not detected. However, inversion of some sucrose as it entered the apoplasm of wt kernels could account for the significant increase in the concentration of hexose in wt compared to that in mn kernels. At 21 DAP, wt kernels have significantly higher sucrose, glucose and fructose specific activity than mn kernels. Since the same sugar pool in the phloem or from the temporary storage sites in the floral rachis (cob) is available to both kernel genotypes, this means that photosynthate partitioning into wt must be faster or greater than into mn kernels. One explanation for this observation is that wt kernels have a stronger driving force for sugar transport from phloem bundles in the kernel pedicel to the endosperm. The presence of invertase activity would support stronger sink strength to attract photosynthates in wt kernels compared to mn kernels. The mn kernels have higher sucrose content than wt kernels at 21 DAP (Figure 4-11 A) probably because mn kernels lack invertase to hydrolyze sucrose to its monomer sugar components. Coincident with these findings, mnl kernels have fewer starch granules and smaller biomass per kernel than wt kernels. Even though the mnl kernels have access to the same assimilate supply as wt kernels, they have fewer endosperm cells and lower starch-synthesis related enzyme activities. It is possible that sugar sensing is the part of only explanation for how the existence of invertase influences sink strength. The absence of the cell-wall bound invertase gene (Mnl) in the mnl mutant leads to the lack of cell-wall bound invertase activity. Without invertase, there is no hydrolysis of sucrose to establish sucrose and hexose concentration gradients from the phloem symplast to P-C apoplast to BETC symplast. If the concentration gradients of sucrose and hexose provide the driving force for sugar transport needed for normal endosperm and kernel
development, a failure to create these gradients could account for the failure of \textit{mn1} endosperms and kernels to fully develop. Some of the transgenic studies done in carrot and potato with introduced exogenous invertase also support the role of sugar gradients for assimilate partitioning and sink strength development (Roitsch et al., 1995; Weber et al., 1996; Herbers and Sonnewald, 1998; Sturm and Tang, 1999). These studies aimed to observe changes in plant development and sucrose partitioning response to a changed sugar environment (sugar composition and quantity). Antisense mRNA for cell wall invertase, vacuolar invertase and sucrose synthase was introduced and expressed in carrot plants (Sturm and Tang, 1999). It was observed that enzyme activities were reduced by more than 90\%, and plants with antisense cell wall invertase mRNA bore no root at all; while the plants with antisense vacuolar invertase and sucrose synthase bore roots of differently reduced sizes (Sturm and Tang, 1999; Tang et al., 1999). These and other studies support the concept that cleavage of sucrose by cell wall invertase at the site of phloem unloading is one of the key steps needed to create a driving force for sink strength development in the storage site of many plants (Herbers and Sonnewald, 1998; Sturm and Tang, 1999).

Sucrose is important for kernel development not only as a carbohydrate source, but it is also thought to play a substantial role as a signaling compound involved in the regulation of certain cellular processes. The former concept is easily understood because of the well-known use of sucrose as a substrate for plant growth. The latter is more complicated and abstruse. Sugar sensing is one of the most novel topics in current plant biology. Sugar sensing and sugar-regulated gene expression in higher plants has become a prominent subject in recent years (Koch, 1996; Jang and Sheen, 1997; Smeekens and Rook, 1997; Chiou and Bush, 1998; Sheen et al., 1999). Data from studies with the \textit{mn} kernel, lacking a sucrose-cleaving enzyme at the base of the endosperm where assimilates enter the filial kernel, provides supporting evidence that interruption of maize endosperm and kernel development is associated with sugar composition and distribution. The maternal pericarp tissue of the \textit{mn} kernel was not affected by the mutation, as no difference in the pericarp mass was found in a preliminary experiment between the two
types of kernels (data not shown). In contrast, endosperm cell division and activities of enzymes associated with starch synthesis were strongly influenced by the mutation. The mn kernels have fewer endosperm cells (Figure 4-4 A), fewer starch granules (Figure 4-4 B) and lower activity of the key starch synthesis enzymes like AGPase and starch synthase than wt kernels (Figure 4-7). It is unknown how the sugar signal leads to the endosperm cells’ response in cell division and further starch synthesis. Since plant hormones and the balance among them are involved in the coordination of cell division and cell differentiation, presumably there is some kind of “cross-talk” between sugar signals and other plant signals, like plant hormones, which will affect cell division more directly. A recent study in carrot showed a linkage between altered sugar partitioning by modified invertase activity and plant cytokinins (Tang et al., 1999). Another study showed that application of exogenous ABA reduced transport of sucrose into wheat grains and lowered the starch synthesis ability of intact grain by altering the activities of enzymes such as AGPase, soluble starch synthase and granule-bound starch synthase activities (Ahmadi and Baker, 1999). Cheng et al. (1999) reported that sugar sensing and the induction of the invertase cell wall gene, INCW1, is independent of the hexo-kinase pathway, suggesting that the 3’ untranslated region of the INCW1 gene acts as a regulatory sensor of carbon starvation and may constitute a link between sink metabolism and cellular translation in plants.

The complexity of hormonal control in plants is displayed by interactions among different classes of plant hormones in the regulation of different developmental and physiological processes. The concentration of each hormone is under the control of poorly understood metabolic regulation responding to a range of signals in plant cells. In addition, it is becoming increasingly evident that these interactions also involve alterations of one hormone level by another (Kaminek et al., 1997). Cytokinins and auxins interact in the control of many developmental processes such as cell division and cell differentiation, organ formation in cultured tissues, apical dominance and leaf senescence. Their involvement in initiation and regulation of cell division and cell elongation underlines the central role of both auxins and cytokinins in plant development.
and the responses of plants to environmental stimuli (Coenen and Lomax, 1997). In maize endosperm, cytokinins have been shown to be involved in early endosperm cell division and auxin in stimulating maize endosperm cell nucleus endoreduplication (Jones et al., 1990; Lur and Setter, 1993a; Dietrich et al., 1995). I suggest that the ratio of cytokinins to ABA and ratio of auxin to cytokinins, rather than levels of these hormones alone, control endosperm cell division and nuclear endoreduplication, respectively (Table 4-1). In this study, there was no difference observed in zeatin and ZR levels between endosperms of the two kernel genotypes during development from 6 to 12 DAP (Figure 4-10). If cytokinin is the only factor determining the cell division and final endosperm cell number, we would expect to see differences in its concentration between wt and mn endosperms, especially in the early developmental stage when cell division is most active. However, if ABA level is also considered and the ratio of cytokinins to ABA is associated with the determination of endosperm cell number, it starts to make sense that the lower ratio of cytokinins to ABA from 6 to 12 DAP leads to inhibited cell division and fewer cells in mn endosperm compared to wt endosperm (Figure 4-4). That mn kernels, which are smaller in size, have higher ABA concentration than wt kernels on a fresh weight basis is similar to previous observations on kernels under heat and water deficit stresses (Ober et al., 1991; Cheikh and Jones, 1994). Maize kernel growth was disrupted or kernels were aborted and they had higher ABA and lower zeatin and ZR concentrations compared to the control kernels when they were treated with heat stress or water deficit stress (Ober et al., 1991; Cheikh and Jones, 1994). Combining the observations of abnormal development of maize kernels under stresses or of the mn mutant, I suggest that the balance of cytokinins and ABA controls cell division in maize endosperm with the higher cytokinin to ABA ratio supporting a more active endosperm cell division. The lower cytokinin to ABA ratio in mn kernels than in wt kernels correlates with the observation of fewer cells in mn endosperm compared to wt endosperm tissue.

The reason for the additional peaks of zeatin and ZR in mn kernels only around 15 DAP is unclear unless the effects of auxin and cytokinin are considered together. In the
growth of maize endosperm, 15 DAP is well beyond the 8 to 10 DAP considered to be the period of most active cell division in this tissue. It is, however, about the time for most active endosperm cell nuclear endoreduplication (Kowles et al., 1990). The timing of active endoreduplication in maize endosperm cells has been shown to coincide with the rapid endosperm cell expansion that occurs from 12 to 16 DAP (Kowles et al, 1990). In wt kernels this endosperm cell expansion correlates well with a dramatic increase in the auxin to cytokinin ratio (Table 4-1). However, in mn kernels, the increase in zeatin and ZR at 15 DAP resulted in a 17 fold lower auxin to cytokinin ratio. The changes in auxin to cytokinin ratio coincided well with the endoreduplication patterns observed in endosperm tissue from kernels on split-pollinated ears. In addition, auxin has been shown to stimulate cell wall loosening and related processes which are involved in expansion growth, but cytokinins have not been shown to have a function in cell enlargement (Kaminek, et al, 1997; Chen, 1998).

In summary, these data are consistent with the concept that invertase at the base of the maize endosperm is a key factor controlling the driving force for assimilate partitioning into maize kernels. The lack of invertase in mn kernels leads to less partitioning (lower biomass, fewer starch granule number per endosperm and abnormal sugar distribution) and disruption of sink development (smaller kernel size, fewer endosperm cells, abnormal endosperm ell nuclear endoreduplication, lower activities of starch synthesis related enzymes and abnormal hormone levels). The mn kernels have fewer endosperm cells and starch granules than kernels with a wildtype phenotype. They also have lower activities of enzymes associated with starch synthesis and they develop smaller kernels than heterozygous kernels with a wildtype phenotype. Endosperm cell DNA endoreduplication and normal distribution of plant hormones were interrupted in mn kernels. The mn kernels had different sugar composition and assimilate partitioning patterns from wildtype phenotype kernels even though they grew on the same ear. I conclude that assimilate partitioning, which results from translocation of photosynthate from source tissue to support sink metabolism and accumulation in sinks, plays an important role in controlling maize kernel sink development. A key mechanism
contributing to the regulation of assimilate partitioning in maize kernels is the expression of genes that control activity of enzymes which regulate sucrose metabolism in the sink tissue.
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