

# Diurnal and developmental changes in levels of nucleotide compounds in developing maize endosperms

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## ABSTRACT

**Maize endosperm is dependent on source tissues to supply the energy and carbon required for development. This supply varies during the course of each day and also throughout development. The impact of these variations on the metabolism of developing endosperm was examined by determining the energy status of the endosperm throughout the course of a day. The adenylate energy charge decreased as the tissue matured, and exhibited a distinct diurnal pattern, reaching a minimum in the afternoon, when the flux of photosynthate is the highest. The minimum value observed was similar to the adenylate energy charge in tissues under mild stress. As the endosperm matured, the adenylate energy charge decreased steadily. The levels of the polysaccharide precursors ADP-glucose and UDP-glucose did not reflect the daily fluctuations in adenylate energy charge, but did exhibit similar long-term behaviour in the latter half of development, decreasing steadily after 21 d after pollination. Similarities in the metabolic patterns of adenylate and uridylate nucleotide levels are discussed in terms of the analogous roles of these compounds in starch and cellulose biosynthesis, respectively. These data provide insight into the metabolic rhythms occurring during endosperm development, and provide a framework for efforts directed toward metabolic engineering.**

*Key-words:* *Zea mays*; adenylate energy charge; endosperm; starch.

## INTRODUCTION

Maize (*Zea mays*) endosperm is the major storage tissue in maize seeds. Its primary function is to accumulate carbon in the form of starch that can be used as an energy source following germination of the seedling. In addition, it is the most important part of the plant from an economical standpoint because it is the major component of the harvested kernels. An understanding of limitations to endosperm development is an important step toward the goal of increasing seed viability or economic value.

During the course of a day, a plant is exposed to a variety

of conditions of temperature and light intensity that produce drastic changes in the ability of the plant to provide photosynthate to the endosperm for storage. It has been shown that the carbon exchange rate and assimilate export rate in leaves both have diurnal patterns that are strongly correlated with irradiance (Kalt-Torres *et al.* 1987). These diurnal patterns in source tissues could result in limitations in the photosynthate supply to developing endosperm, which could impact the rate of assimilate storage by endosperm tissue.

The energy status is a good indicator of the overall metabolic condition of a cell. The adenylate energy charge is a measure of the energy status, and is based on the proportion of adenylate nucleotides in the di- and tri-phosphate form. A convenient expression for adenylate energy charge has been proposed  $[(ATP) + 0.5(ADP)] / [(ATP) + (ADP) + (AMP)]$  where AMP, ADP, ATP are adenosine 5'-mono-, -di- and -triphosphate, respectively (Atkinson & Walton 1967). Expressed in this way, adenylate energy charge varies from 1 (all adenylate nucleotides in triphosphate form) to zero (all adenylate nucleotides in monophosphate form). Actively metabolizing tissues typically have adenylate energy charge values above 0.8. Because it correlates with several stress conditions, adenylate energy charge has been used as a measure of stress (Ivanovici & Weibe 1981). In oxygen-stressed germinating seeds, for example, the adenylate energy charge has been shown to be as low as 0.3 (Raymond & Pradet 1980).

Because two of the major activities of developing endosperm are synthesis of starch and cellulose, the levels of precursors of these compounds can be thought of as metabolic indicators as well. The nucleotide sugars ADP-glucose and UDP-glucose are involved in the biosynthesis of starch and cellulose, respectively. ADP-glucose is produced by ADP-glucose pyrophosphorylase in the rate-limiting step in starch biosynthesis (Stark *et al.* 1992). UDP-glucose metabolism is less well characterized, but an increasing body of evidence suggests that it is the precursor to cellulose (Pear *et al.* 1996).

The aim of this study was to provide an overview of the metabolic state of developing endosperm by monitoring the energy status and the levels of key metabolic precursors. Because these parameters are fundamental to endosperm metabolism, this information will provide a framework for interpretation of other metabolic data.

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## MATERIALS AND METHODS

### Plant growth and tissue collection

For the diurnal study, the maize inbred OH43 was grown at the Iowa State University Agronomy Farm in the summer of 1998. Ears to be sampled were self-pollinated. At each time point, the husks were peeled back and about 10 endosperms were dissected from the attached ear and immediately frozen in liquid nitrogen. The husks were replaced until the next sampling. The frozen endosperms were wrapped in foil and stored at  $-80^{\circ}\text{C}$  until use.

For the developmental study, self-pollinated ears of field-grown OH43 were grown in 1999 and were harvested and taken to the laboratory on ice before removing the husks and dissecting the kernels. Endosperms were removed and stored as previously described. To minimize diurnal effects on the measurements, ears were harvested in the early afternoon on each day of the study. Readers should use caution when comparing data between the diurnal study and the developmental study because the plants used in these two studies were produced under different environmental conditions.

### Extraction of nucleotide compounds

Nucleotide compounds were extracted using the method of Shannon, Pein & Liu (1996) with the following modification. About 0.5 g of frozen endosperms were weighed and homogenized in 2 mL 0.8 M  $\text{HClO}_4$  using a rotor-stator homogenizer (Kinematica, Lucerne, Switzerland). Neutralization, centrifugation and washing of the pellets were carried out as described (Shannon *et al.* 1996). To determine the efficiency of extraction, samples were spiked prior to extraction with a known amount of each nucleotide compound being investigated. The amount of each compound in the spiked samples was then compared to the amount of each compound in a duplicate sample lacking the spike. At each time point in the diurnal study the endosperms from each of two or three ears (at 22 and 18 d after pollination, respectively) were extracted separately. A similar

procedure was used in the developmental study, except that endosperms from a single ear were extracted in triplicate for each date in the study.

### High-performance liquid chromatography analysis of nucleotide compounds

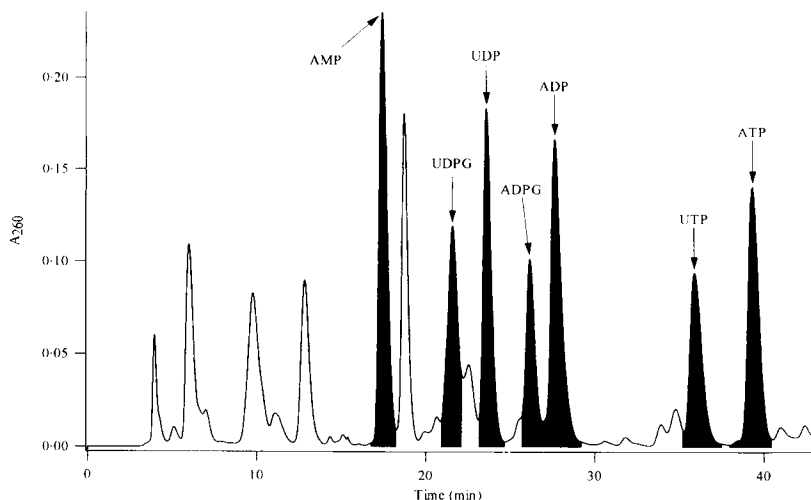
High-performance liquid chromatography (HPLC) analysis was conducted as described previously (Shannon *et al.* 1996). Extracted nucleotide compounds were separated on a PerSeptive Biosystems Biocad Sprint HPLC (Applied Biosystems, Foster City, LA, USA) fitted with an Alltech (Deeffield, IL, USA) Nucleotide/Nucleoside column and a Timberline Instruments (Boulder, CO, USA) TL-30 column heater operating at  $40^{\circ}\text{C}$ . The mobile phase consisted of a gradient from 95% solvent A/5% solvent B to 70% solvent A/30% solvent B where solvent A was 20 mM  $\text{KH}_2\text{PO}_4$ , 5 mM tetrabutyl ammonium phosphate and solvent B was HPLC grade methanol. The UV absorbance of the column effluent was monitored at 260 nm. Peaks were identified by comparing retention times with those of commercially obtained standard compounds (Sigma, St Louis, MO, USA). The UV-visible absorbance spectrum of each peak was compared with that of the comigrating standard compound as well.

Peaks were quantified by integration using the program IgorPro (Wavemetrics Inc., Oswego, OR, USA). The area under each peak (corresponding to the shaded areas in Fig. 1) was converted to the absolute amount of each compound using a standard curve constructed by injecting three different amounts of each standard compound on the column and integrating the resulting peaks in the same way as the samples under investigation.

## RESULTS

### Levels of extractable nucleotide compounds in developing endosperm

Diurnal patterns have been observed in the carbon exchange rate and assimilate export rate in maize leaves



**Figure 1.** A typical profile of acid extractable nucleotide compounds from maize endosperm separated by HPLC and visualized with UV absorbance detection. The shaded portions of the chromatogram were integrated to quantify each of the compounds shown.

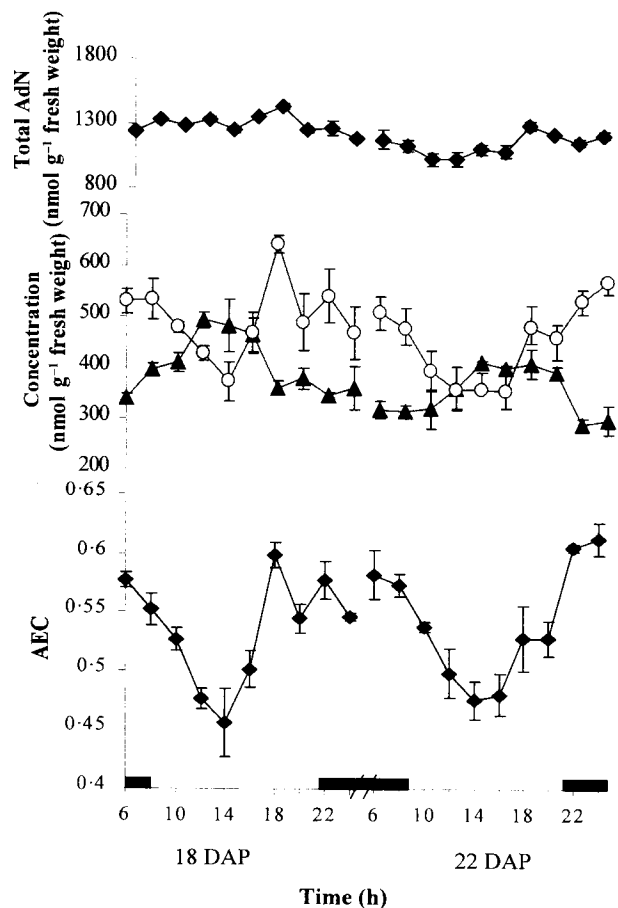
(Kalt-Torres *et al.* 1987). Endosperm metabolism is tied to leaf metabolism by the source-sink relationship, so it seems likely that the diurnal patterns in leaves are somehow reflected in corresponding patterns in endosperm. Nucleotide compounds play key roles in endosperm metabolism. They provide energy and reducing potential for cellular metabolism, as well as metabolic precursors for polysaccharide biosynthesis. Given their central metabolic role, these compounds can be considered indicators of the metabolic state of endosperm tissue.

We characterized the nucleotide compounds in developing maize endosperm using an acid extraction followed by HPLC separation with UV detection (Shannon *et al.* 1996). This method allowed us to quantify the levels of ATP, ADP, AMP, UTP, UDP, UDP-glucose and ADP-glucose, where UDP and UTP are uridine 5'-di- and -triphosphate, respectively (Fig. 1). The efficiency of recovery of nucleotide compounds using this method has been documented (Shannon *et al.* 1996) but this experiment was repeated to verify that similar efficiencies were being obtained. We also wanted to know if the developmental stage of the tissue affected the recovery. At 18 d after pollination, the recovery of the compounds under investigation was as follows: AMP 106%, ADP 107%, ATP 111%, UDP 112%, UTP 111%, UDPG 108% and ADPG 121%. At 30 d after pollination the recovery of the compounds under investigation was as follows: AMP 108%, ADP 113%, ATP 106%, UDP 107%, UTP 105%, UDPG 107% and ADPG 109%. The difference in recovery from samples harvested at 18 d after pollination and samples harvested at 30 d after pollination was not significant.

In all samples, the adenyl and uridyl nucleotides were the most abundant, typically accumulating to 100–600 nmol g<sup>-1</sup> fresh weight. The nucleotide sugars ADP-glucose and UDP-glucose were the next most abundant at about 150–300 nmol g<sup>-1</sup> fresh weight. When these peaks were not resolved to the baseline (see Fig. 1), arbitrary peak limits were set. This may introduce error into the determination of the absolute amounts of these compounds, but because the same integration limits were used within a given data set, comparisons within data sets should be valid. Several unidentified peaks had similar magnitudes to the compounds under investigation. These results are similar to those observed previously for similar tissues (Shannon *et al.* 1996).

### Diurnal pattern of adenylate energy charge

By examining nucleotide composition data from endosperms dissected at intervals of 2 h during the course of the day, we were able to identify diurnal patterns in the levels of these metabolites. The adenylate energy charge was calculated from these data (Atkinson & Walton, 1967). Adenylate energy charge reached a minimum of about 0.48 in the afternoon when temperature and light levels were highest, and returned to about 0.68 at night. A similar pattern was observed on two different days, at 18 and 22 d after pollination (Fig. 2, bottom panel). Light and temper-



**Figure 2.** Diurnal changes in adenylate nucleotide levels in developing maize endosperm. Nucleotides were extracted from maize endosperms harvested at intervals of 2 h and were quantified by HPLC. The top panel shows the total adenylate nucleotide level, AMP + ADP + ATP. The middle panel shows the levels of ATP (○) and AMP (▲). The adenylate energy charge (AEC) was calculated from the concentrations of AMP, ADP and ATP. Error bars delimit one standard deviation from the mean of two measurements at 22 d after pollination (DAP) and three measurements at 18 DAP.

ature conditions were similar on these two days. This pattern in adenylate energy charge is largely due to reciprocal changes in the size of the pools of ATP and AMP (Fig. 2, middle panel). The concentration of ATP was lowest in the afternoon when the concentration of AMP was highest. The total adenylate nucleotide pool (ATP + ADP + AMP) did not vary substantially in the course of a day (Fig. 2, top panel). Given the diurnal patterns in source tissue metabolism (Kalt-Torres *et al.* 1987), it is not surprising that a diurnal pattern existed in a sink tissue as well.

### Levels of starch and cellulose precursors in the course of a day

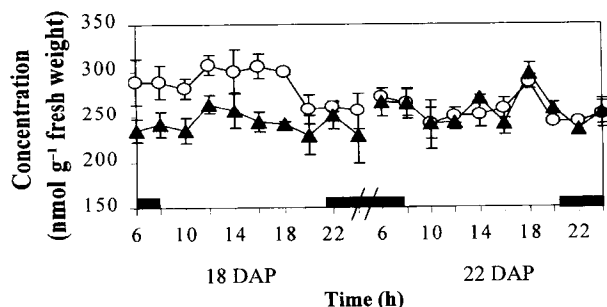
A main function of endosperm tissue is to accumulate starch and cellulose for storage and structural purposes, respectively. The nucleotide sugars ADP-glucose and UDP-

glucose are involved as activated monomers that are polymerized in polysaccharide formation. Both of these compounds require substantial energy for their synthesis. ADP-glucose pyrophosphorylase has a major role in ADP-glucose metabolism, and UDP-glucose pyrophosphorylase may play a similar role in UDP-glucose metabolism. ATP and UTP, respectively, are substrates for these enzymes. For these reasons, we wanted to see if the change in the energy status of the cell was reflected in a change in the levels of the polysaccharide precursors ADP-glucose and UDP-glucose. These compounds were quantified using the same HPLC runs used to quantify the adenylate nucleotides (Fig. 1). In spite of the close relationship between energy metabolism and sugar nucleotide metabolism, ADP-glucose and UDP-glucose did not exhibit a diurnal pattern reflecting the pattern observed for adenylate energy charge. Rather, both nucleotides fluctuated between 200 and 300  $\text{nmol g}^{-1}$  fresh weight without any clear pattern (Fig. 3).

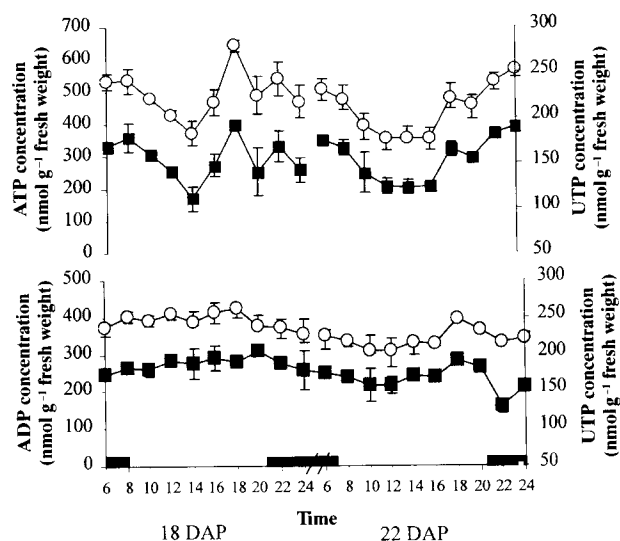
It is important to consider that steady-state levels of ADP-glucose and UDP-glucose were being measured, so the relatively invariant levels of these compounds did not necessarily indicate a constant flux through a pathway. These data indicated that the metabolic pools of these compounds were not substantially altered during the course of a day.

#### Diurnal pattern of UTP and UDP content parallels that of ATP and ADP

ADP-glucose and UDP-glucose have similar roles as polysaccharide precursors. ATP provides the energy required for starch metabolism, so it is possible that UTP has an analogous role in the biosynthesis of UDP-glucose-derived polysaccharides. If this were the case, the patterns of UTP levels could be similar to those of ATP. Examination of our data revealed that UTP levels were highly correlated with ATP levels (Fig. 4). Like ATP, UTP exhibited a diurnal



**Figure 3.** Level of ADP-glucose (○) and UDP-glucose (▲) in developing endosperm throughout the course of two days. Error bars delimit one standard deviation from the mean of two measurements at 22 d after pollination (DAP) and three measurements at 18 DAP.



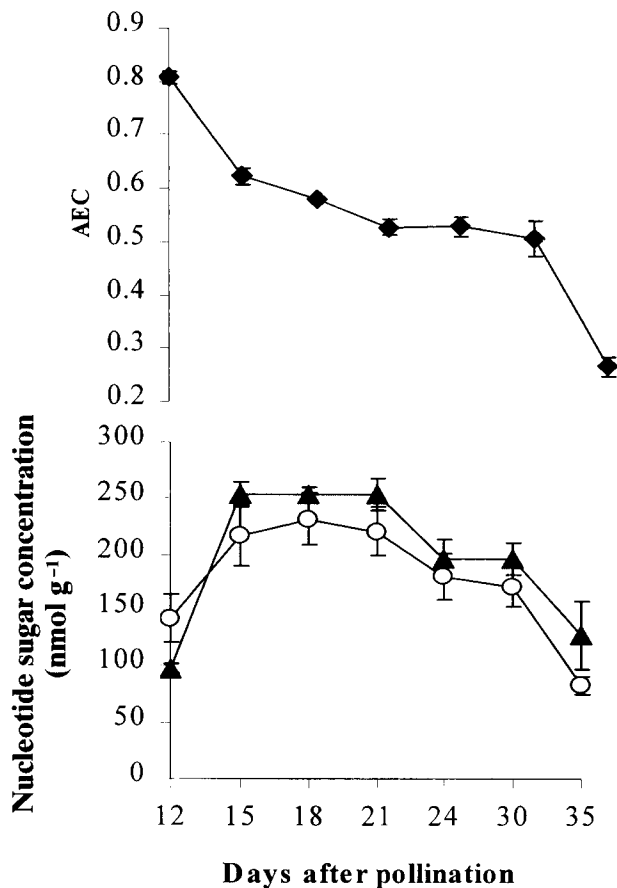
**Figure 4.** Concentrations of UTP (top panel, ■), ADP (bottom panel, ○) and UDP (bottom panel, ■) over the course of two days. Data for ATP (top panel, ○) is the same as in Fig. 2, and is shown for comparison. Error bars delimit one standard deviation from the mean of two measurements at 22 d after pollination (DAP) and three measurements at 18 DAP.

pattern that reaches a minimum at 1400 h. In contrast to ATP, UTP, and AMP, the levels of ADP and UDP did not exhibit a distinct diurnal pattern (Fig. 4). The observations that both the adenylate and uridylylate triphosphate nucleotides exhibit a diurnal pattern whereas both diphosphate nucleotides did not established an analogy between adenylate nucleotides and uridylylate nucleotides in metabolism.

#### Developmental pattern of adenylate energy charge

In dormant seeds, the adenylate energy charge has been determined to be 0.21 and it rapidly rises to above 0.8 upon germination (Rodaway, Huang & Marcus 1979). Given the higher values of adenylate energy charge in immature seeds, the low value in dormant seeds suggests that the energy charge must be reduced during the course of seed development. To ascertain the details of this reduction, the adenylate energy charge in developing endosperms was measured at different stages of development (Fig. 5, top panel). It was observed that the energy charge decreased at a fairly constant rate of about 0.03/d.

These results were different from observations of the energy charge of developing soybean seeds (Quebedeaux 1981). Whereas a continuous decrease in adenylate energy charge in corn endosperm was observed, in soybeans a distinct plateau was observed during mid-maturation. This difference could be the result of physiological differences between seed development of monocots and dicots.



**Figure 5.** Developmental pattern of nucleotide levels. Samples were harvested at the same time of day to avoid confounding the diurnal and developmental effects. The top panel illustrates the adenylate energy charge whereas the bottom panel illustrates changes in the polysaccharide precursors ADP-glucose (open circles) and UDP-glucose (filled triangles). Error bars delimit one standard deviation from the mean of three measurements.

### Developmental pattern of starch and cellulose precursors

The levels of ADP-glucose and UDP-glucose remained roughly parallel in the course of development (Fig. 5, bottom panel), again emphasizing the parallel roles these compounds play in endosperm development. Both compounds increased to a maximum that extended from 15 to 21 d after pollination, followed by a gradual decrease through the rest of development. This pattern is parallel to that of the adenylate energy charge in the latter half of development. This is in contrast to the diurnal study, in which the sugar nucleotide levels were unrelated to the adenylate energy charge. Thus it appears that sugar nucleotide levels do not mirror daily fluctuations in adenylate energy charge, but long-term changes in sugar nucleotide levels follow a similar pattern to adenylate energy charge in the latter part of development.

### DISCUSSION

The aim of this study was to gain an overview of energy metabolism in developing endosperm. This was achieved by monitoring levels of key metabolites during the course of a day and through the course of development.

In maize leaves, the carbon exchange rate, assimilate export rate and sucrose concentration are all maximal in the afternoon (Kalt-Torres *et al.* 1987) when the adenylate energy charge was observed to be at a minimum. The adenylate energy charge may drop at this time as a result of the energy expenditure required to convert the high levels of photosynthate to a storable form such as starch or cellulose. At night, when the flux of photosynthate is lower, normal cellular metabolism is capable of restoring the energy charge.

Taken together, these data support several interesting conclusions about endosperm development. The diurnal variations in energy charge are relatively large, reaching minimum values equivalent to those of tissues under stress. In spite of this fluctuation in energy charge, levels of the key polysaccharide precursors are maintained at a fairly constant level. This underscores the importance of these compounds in fulfilling the primary role of this tissue, which is storage of carbon for use by the germinating seed. One possible interpretation of these data is that the mechanisms for storing carbon have metabolic priority over those mechanisms for maintaining the energy level of the cell. Rather than metabolizing the incoming carbon to maintain the energy level of the cell, this carbon is used to maintain levels of polysaccharide precursors so that polysaccharide synthesis can continue.

The diurnal variation in adenylate energy charge is most likely a response to changing environmental conditions such as light intensity and/or temperature. At this point we cannot distinguish between diurnal variation and a circadian rhythm. These issues could be addressed in carefully designed experiments using growth chambers in which light and temperature are controlled.

A second striking feature of the data is the parallel behaviour of the adenylate and the uridylylate nucleotides. Similar diurnal patterns were observed in the levels of the ATP and UTP, ADP and UDP, and ADP-glucose and UDP-glucose. These similar patterns could indicate that these compounds play parallel roles in metabolism. ADP-glucose and therefore ATP are considered to play important roles in starch biosynthesis, whereas UDP-glucose and therefore UTP are considered to play important roles in cellulose biosynthesis. It may therefore be useful to consider analogies between starch and cellulose biosynthesis when developing hypotheses to explain the biosynthetic mechanisms of these polysaccharides.

Our data are valuable because they provide an overview of the concentrations of nucleotide compounds at the tissue level. It is important to consider the subcellular localization of metabolites and the enzymes utilizing them when assessing the roles of these compounds in polysaccharide biosyn-

thesis. Different forms of ADP-glucose pyrophosphorylase are present inside and outside of plastids, for example (Denyer *et al.* 1996; Thorbjørnsen *et al.* 1996) and alterations in putative nucleotide transporters affect nucleotide levels (Shannon *et al.* 1996) or the physiology of starch production (Tjaden *et al.* 1998). It would be informative to know the levels of these compounds in different subcellular compartments.

These results have important implications for efforts directed to altering the metabolic pathways of endosperm tissue. When considering metabolic engineering of endosperm tissue, it is important to realize that the highest metabolic capacity of the tissue from an energetic standpoint is early in development and perhaps surprisingly, during the night. Efforts to produce novel products in seeds may benefit by taking this information into account. If the synthesis of the novel products is energy intensive, but not heavily dependent on a large influx of carbohydrate, for example synthesis of a protein product, it may be better to engineer the novel pathway to be most active at night and in early development.

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