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Starch synthesis and carbon partitioning in developing endosperm

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Abstract

The biosynthesis of starch is the major determinant of yield in cereal grains. In this short review, attention is focused on the synthesis of the soluble substrate for starch synthesis, ADPglucose (ADPG). Consideration is given to the pathway of ADPG production, its subcellular compartmentation, and the role of metabolite transporters in mediating its delivery to the site of starch synthesis. As ADPG is an activated sugar, the dependence of its production on respiration, changes which occur during development, and the constraints which ATP production may place on carbon partitioning into different end-products are discussed.

Key words: ADPglucose, carbon partitioning, cereals, development, endosperm, starch.

Introduction

Wheat (*Triticum* sp.) has been grown throughout temperate regions of the world since prehistoric times. Species of wheat are classified according to the number of chromosomes found in the vegetative cell. They are divided into three species: the diploid containing 14 chromosomes (e.g. *Triticum speltoides*, *T. bicorne* and *T. tauschii*), the tetraploid containing 28 chromosomes (e.g. *T. timopheevii* and *T. turgidum*) and the hexaploid containing 42 chromosomes (e.g. *T. aestivum*).

A large proportion of man's essential nutrients are contained in the wheat grain (60–80% carbohydrate, 8–15% protein, 1.5–2% fats, 1.5–2% minerals and vitamins). The majority of the carbohydrate portion is attributed to starch which constitutes approximately 70% of mature grain dry weight (Dale and Housley, 1986). In addition to its high nutritive value, the low water content, ease of transport and processing, and good storage qualities have made this crop the most important staple food of more than one billion people (35% of the world's population).

In the UK during 2000, 16.7 million tonnes of wheat was harvested, accounting for approximately 70% of the total cereal produced (MAFF figures, 2000, http://www.maff.gov.uk). Clearly, the endosperm is the major site of storage product deposition and is the focus of the current review. However, any understanding of the regulation of carbon partitioning has to be viewed in the context of endosperm development during grain formation.

Seed development

Wheat grains consist of the seed coat, or testa, which surrounds the endosperm and embryo. The endosperm, the largest organ in the seed is surrounded by a single layer of cells, the aleurone layer. The embryo is comprised of the embryonic axis (incorporating the embryonic root and the hypocotyl), a single cotyledon, containing the first true leaves, and the scutellum. Seed development commences after fertilization of the ovule, which, in the angiosperms, involves the participation of two male nuclei. One nucleus fuses with the egg forming a diploid zygote and gives rise to the embryo, the other fuses with two polar nuclei to produce a triploid nucleus, further division of which produces the endosperm.

Cereal grain development can be divided into two main stages, grain enlargement and grain filling. The first stage, grain enlargement, involves early, rapid division of the zygote and triploid nucleus. Cell division is followed by

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the influx of water, which drives cell extension. This stage occurs at approximately 3–20 d post-anthesis (dpa) (Briarty *et al*., 1979). During the second stage (grain filling), cell division slows and then ceases and storage products are accumulated, beginning at around 10 dpa until maturity when the endosperm serves its function as a carbohydrate store (Briarty *et al*., 1979; Bewley and Black, 1994).

Endosperm development

Briarty *et al*. (1979) have carried out an extensive morphological analysis of changes which occur during endosperm development. Figure 1 is a summary of the major changes which take place and shows the relationship between protein and starch deposition, and cell wall synthesis. The data (recalculated from Briarty *et al*. (1979)) are based on a stereological analysis of the volumes occupied by each component within the endosperm cell. Whilst they cannot be related to one another in terms of dry matter, and will be influenced by water content which changes during development, the data give a good indication of the major changes which are occurring, and their timing. Amyloplasts, involved in starch synthesis and storage, increase in number per cell. Indeed the volume of the cells occupied by starch increases throughout development, reaching 65% at 36 dpa. The starch of wheat endosperm is composed of two types of granule, the synthesis of which appears to be developmentally regulated. The larger, A-type granules, appear approximately 4 d earlier in development than the smaller, B-type granules (~22 dpa), and are contained in A-type and B-type amyloplasts, respectively. The rough endoplasmic reticulum (RER), involved in the synthesis of proteins, was shown to increase in surface area per cell, and surface–volume ratio, between 10–12 dpa, around about the time that protein deposits are observed. Interestingly, Briarty *et al*. (1979) also observed that the mitochondrial number per cell increases during development, but that the individual mitochondrial volume decreases, as does the area of the inner membrane per unit volume of total cytoplasm. The possible implications of such structural changes on metabolism within endosperm cells during development will be discussed later.

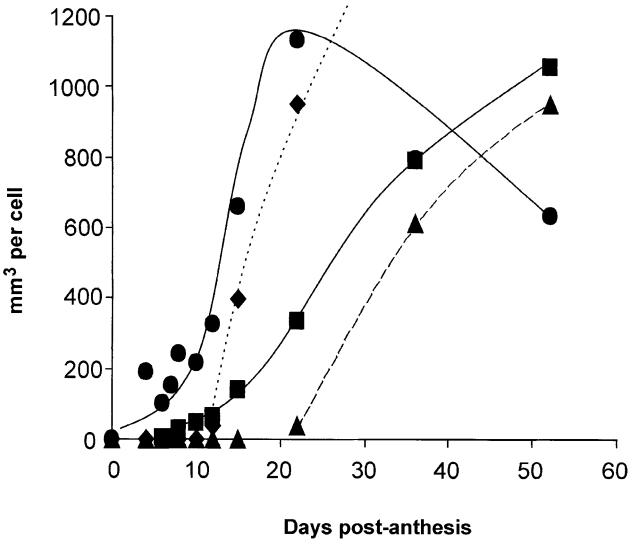

Fig. 1. Changes in components of wheat endosperm during development. Data are expressed as volumes (μm^3) and are based on a stereological analysis reported by Briarty *et al*. (1979). (filled circles) Cell wall ($\times 10^{-1}$), (filled diamonds) protein ($\times 10^{-1}$), (filled squares) A-type starch granules ($\times[0.5\times 10^{-2}]$), (filled triangles) B-type starch granules ($\times 10^{-2}$).

Carbohydrate partitioning and starch synthesis in developing endosperm

The main carbohydrate transported in higher plants is sucrose. The phloem provides a means for long-distance movement. However, cereal grains have no direct vascular connections with the parent plant and so a short-distance transport mechanism operates to move sucrose from vascular tissues to the endosperm. Wheat grains possess a furrow running along the length of the kernel with a vascular bundle embedded at the bottom. Nutrient unloading occurs along the length of the bundle, and has to pass through three distinct layers before reaching the inside of the grain. Solutes are unloaded from the phloem symplastically through the chalaza and then into the apoplast via the specialized transfer cells in the nucellus, before it reaches the outer layer of the endosperm where transfer cells in the aleurone layer redirect solutes back into the symplasm and into endosperm cells (Thorne, 1985).

The pathway of starch synthesis in non-photosynthetic storage tissue involves the conversion of sucrose into ADPglucose (ADPG), and the subsequent conversion of this soluble precursor into insoluble polyglucan (Fig. 2). The enzymes involved in the synthesis of starch from ADPG are the starch synthases, branching enzymes and debranching enzymes, and are located exclusively within plastids. Their role and regulation has been reviewed extensively elsewhere (Smith, 1999; Smith *et al*., 1997, 2003) and are not the main consideration of this article.

Sucrose synthase (SuSy) is generally considered to catalyse the first step in the conversion of sucrose to starch in the endosperm of the grain. In wheat grains, SuSy activity is primarily associated with the endosperm, with the highest activities occurring during periods of peak starch synthesis (Dale and Housley, 1986). SuSy exists in both cytosolic and membrane-bound forms. Activity of the cytosolic form is correlated with the production of storage products, such as starch, and respiration (Chourey and Nelson, 1976; Xu *et al*., 1989), whereas the membrane-

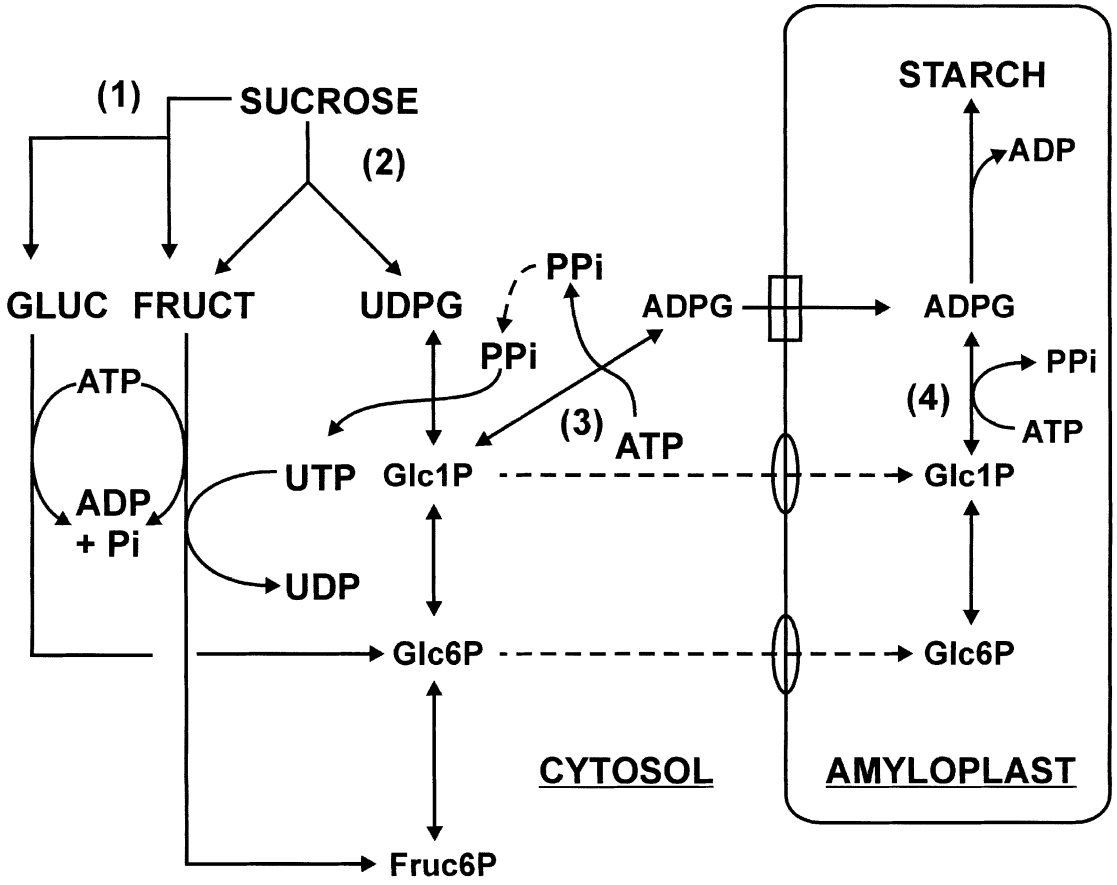

Fig. 2. Interconversion of sucrose to starch in developing endosperm. Enzymes shown are (1) invertase, (2) sucrose synthase, (3) cytosolic AGPase, and (4) plastidic AGPase.

bound form is believed to be involved in cellulose and callose synthesis (Amor *et al*., 1995). At the present time there is no clear evidence for a role for invertase on the breakdown of sucrose in endosperm. The UDPglucose and fructose formed by SuSy may then be converted to hexosephosphates for import into amyloplasts, or to ADPglucose in the cytosol, prior to the uptake of activated sugar (see below).

Synthesis of ADPglucose

The enzyme responsible for the production of ADPG is ADPglucose pyrophosphorylase (AGPase), which has been studied extensively (Preiss and Sivak, 1996). In many species the enzyme is sensitive to allosteric regulation by phosphoglyceric acid (activator) and inorganic orthophosphate (inhibitor). Whilst this appears to be the case for the enzyme in leaf tissue, and also storage tubers of dicots such as potato, there is an increasing body of evidence which suggests that the activity in some cereal endosperms is much less sensitive to such regulation. A recent report demonstrated that an AGPase, purified from wheat endosperm was insensitive to activation by PGA (Gómez-Casati and Iglesias, 2002) although PGA could reverse inhibition by Pi. However, the above study did not demonstrate the subcellular localization of this form of the enzyme, a point which is seen as increasingly pertinent in the study of the enzyme in cereals.

For many years, it was generally accepted that AGPase is exclusively located in plastids of one type or another, be they chloroplasts or amyloplasts. There is now good reason for believing that this does not hold true in cereal endosperm. In the developing endosperms of maize and barley, there is a cytosolic isoform which accounts for 85–95% of the total activity (Denyer *et al*., 1996; Thorbjørnsen *et al*., 1996). The enzyme is a heterotetramer, consisting of large, AGP-L, and small, AGP-S, subunits (the latter bearing the catalytic activity). Doan *et al*. (1999) have expressed both barley endosperm subunits in baculovirus, separately and in combination. Whilst AGP-S was sensitive to activation by PGA, when AGP-S was expressed alongside AGP-L, this sensitivity was lost. Further, this PGA-insensitive form of the enzyme has been shown to be extraplastidic, lacking any identifiable transit peptide necessary for targeting to amyloplasts. Expression of the barley endosperm cytosolic AGPase

subunits appears to be developmentally regulated, with both large and small subunit mRNAs appearing at around 10–12 dpa (Doan *et al*., 1999). The equivalent subunits have also been cloned from wheat endosperm (Ainsworth *et al*., 1993, 1995) with AGP-S being expressed earlier in endosperm development than AGP-L. Although the cDNAs cloned by Ainsworth *et al*. were originally thought to code for plastidic subunits, alignment of the sequences with the barley subunits indicates that they are virtually identical. Wheat endosperm, like barley and maize, appears to contain AGPase activity in both the cytosol and amyloplast (Vardy *et al*., 2002).

Beckles *et al*. (2001) reasoned that because of the presence of UDPglucose (UDPG) pyrophosphorylase in the cytosol, the ratio of ADPG to UDPG would be higher in tissues expressing a cytosolic AGPase, compared with species where UGPase and AGPase are in discrete subcellular compartments. A thorough analysis of a large number of mono-and dicotyledonous species showed that the ratio of ADPG:UDPG was generally an order of magnitude higher in cereal endosperms compared to other species/organs (Beckles *et al*., 2001), and also concluded that there was no evidence for a cytosolic AGPase in non-graminaceous species. The dual location of AGPase in different subcellular compartments has implications for both the regulation of starch synthesis, the pathway of which is therefore bifurcated, and the location and movement of metabolites into amyloplasts (Fig. 2).

Transport of metabolites for starch synthesis into amyloplasts

The possibility exists, therefore, that ADPG may be synthesized in both the cytosol and the amyloplast of wheat/cereal endosperm. Synthesis within the organelle requires a supply of hexosephosphate and ATP. Flügge and co-workers (Kammerer *et al*., 1998) have cloned a hexosephosphate:Pi exchanging carrier from a number of species and were able to express a functional protein, heterologously, corresponding to that found in the envelopes of pea root plastids. Whilst this protein was able to bind glucose 6-phosphate (Glc6P), it was unable to transport glucose 1-phosphate (Glc1P). However, the reconstitution of envelope proteins from wheat endosperm amyloplasts into proteoliposomes demonstrated the presence of a protein which was able to catalyse the counter exchange of either Glc6P or Glc1P with orthophosphate (Tetlow *et al*., 1996). The hexosephosphates were competitive inhibitors with each other, suggesting a common carrier and leaving open the possibility that either could be imported from the cytosol to support starch synthesis. Studies of starch synthesis in isolated amyloplasts, suggested that Glc1P is a better precursor for starch synthesis than Glc6P (Tetlow *et al*., 1994), although this observation must be mitigated by the relative *in vivo* concentrations of the two hexosephosphates, where Glc6P tends to be in 10–20-fold excess of Glc1P because of the equilibrium constant of phosphoglucomutase. Whichever hexosephosphate is transported, there is a requirement for ATP within the amyloplast. Whilst this could be generated by oxidative metabolism within the organelle (Plaxton, 1996), studies with potato tubers suggest that a plastidic ATP/ADP transporter protein (AATP) is more significant in regulating starch synthesis when ADPG is synthesized within amyloplasts (Kampfenkel *et al*., 1995; Tjaden *et al*., 1998; Geigenberger *et al*., 2001). Neuhaus and his co-workers have demonstrated that this protein is distinct from its mitochondrial counterpart, and has a profound effect on starch synthesis. Decreasing AATP activity in potato tuber by antisense technology caused a reduction in starch synthesis, whilst increased activity (caused by heterologous expression of AATP from *Arabidopsis thaliana*) led to an increase in starch synthesis and tuber yield. Further, the activity of AATP also influenced the ratio of amylose:amylopectin by altering the content of ADPG. Amylose synthesis is relatively more favoured at higher ADPG concentrations, as the affinity of the granule-bound starch synthase, which is responsible for amylose synthesis, is about 10-fold lower than that of the soluble starch synthases which contribute to amylopectin synthesis (Smith *et al*., 1997). Thus, in transgenic potato tubers, as the activity of AATP increased, so also did the content of ADPG and the ratio of amylose:amylopectin (Tjaden *et al*., 1998; Geigenberger *et al*., 2001). There are no published reports of this transporter in monocots, though a partial cDNA with a high degree of homology to the *Arabidopsis* protein has been obtained from wheat endosperm (HE Neuhaus, personal communication).

It seems that in cereal endosperm, the bulk of AGPase activity is associated with the cytosol (see above). Consequently, there is a requirement to transport ADPG into the amyloplast which cannot be met by the AATP referred to previously, as this is highly specific for ATP and ADP and shows no affinity towards nucleotide sugars. The *Bt1* locus of maize encodes a protein of 38–42 kDa localized in amyloplast membranes (Sullivan and Kaneko, 1995) and is believed to play a role in the transport of ADPG (Shannon *et al*., 1998). Recent studies in the authors' laboratory have demonstrated the presence of an ADPG transporter in wheat endosperm amyloplasts (Emes *et al*., 2001). A 38 kDa protein from envelope preparations has been solubilized in detergent and reconstituted into artificial membrane systems. The protein is able to catalyse the counter exchange of ADPglucose with AMP, ADP or ATP, but does not bind UDPglucose or other uridylates (M Emes, unpublished observations). The ADPG transporter can be identified in wheat endosperm by covalent cross-linking to radiolabelled azido-ADPglucose. The data shown in Fig. 3 suggest that it is not readily detected until 10 dpa and that content increases subsequently,

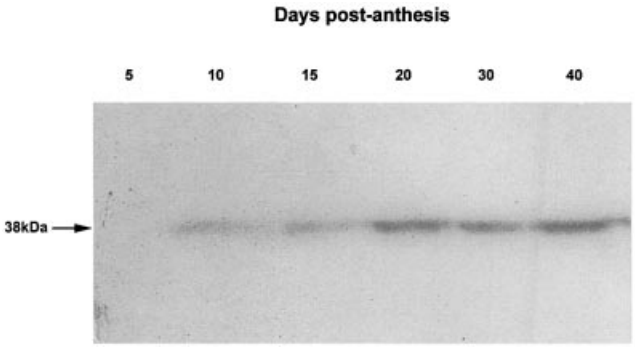

Fig. 3. Changes in content of ADPglucose transporter protein during wheat endosperm development. Membranes were isolated from whole cell extracts of endosperm and incubated with 5 mM N_3-[α-^{32}P] ADPglucose for 1 min at 25 °C, followed by cross-linking under uv light. Membrane proteins were separated by 10% SDS-PAGE and photolabelled protein visualized by autoradiography. Each lane was loaded with 25 μg protein.

coinciding with the major period of grain-filling and consistent with the changes observed in the expression of cytosolic AGPase in barley endosperm (Doan *et al*., 1999). Given that this protein is possibly the major route for the import of the soluble substrate of starch synthesis, ADPG, it is likely that its activity will also have an impact on the balance of amylose and amylopectin synthesis in the same way as has been observed for the potato AATP (see above).

Turnover of ADPglucose

Evidence has recently been provided for a further component which may regulate the ADPglucose content of starch synthesizing tissues. Pozueta-Romero and co-workers (Rodríguez-López *et al*., 2000) have provided evidence for the existence of an enzyme which specifically breaks down ADPG, ADPglucose pyrophosphatase (AGPPase), catalysing the reaction:

$$\text{ADPGlucose} \rightarrow \text{Glc1P} + \text{AMP}$$

Unlike AGPase, AGPPase is a 35 kDa monomeric protein which is inhibited by PGA as well as Pi, PPi, ATP, and ADP. The enzyme was found in both the cytosol and plastids of barley endosperm with about 30% of the total activity being accounted for inside amyloplasts. The activity of AGPPase was highest just after pollination (8 dap), and had decreased to a minimal level by 20 dap. The enzyme was also found in several other species including wheat leaves and endosperm, tomato leaves, sycamore cell cultures, potato tubers, and *Arabidopsis* leaves. The presence of such an enzyme in endosperm tissue suggests that the regulation of ADPG content in either the cytosol or amyloplast may include a further level of complexity. The observation that AGPPase activity declines markedly during endosperm development suggests that it is regulated developmentally to a level consistent with the role of this tissue as a storage organ.

Respiratory changes in relation to starch synthesis during endosperm development

In whichever compartment ADPglucose is synthesized, the fact remains that there is a requirement for ATP during starch synthesis which will be a minimum of one ATP for each mole of sucrose converted to starch (this value assumes turnover of PPi, ATP and UTP between AGPase and UDPglucose pyrophosphorylase in the cytosol). To date, there have been few studies in which the synthesis of starch in cereal endosperm has been considered in relation to the energetic requirements of storage product deposition. Respiration in wheat endosperm was measured together with the activities of citrate synthase and cytochrome oxidase. The activity of these enzymes decreased by 85% between 6–10 dpa and respiration, measured as O_2 consumption, fell by 60% from 0.37 μmol min^{-1} g^{-1} FW to 0.15 μmol min^{-1} g^{-1} FW (data not shown) consistent with the observation that there is a decrease in the density of cristae in mitochondria between 4–16 dpa (Briarty *et al*., 1979). The rate of cell division decreases during the early to mid-stage (8–14 dpa) of development and ceases by approximately 20 dpa depending on growth conditions, cultivar etc (Briarty *et al*., 1979). Implicit in this observation is that rapidly dividing cells require more ATP and carbon skeletons than enlarging or fully differentiated cells, as has been observed in developing potato tubers (Appeldoorn *et al*., 1999). The rate of respiration during the main period of grain filling, from 10 dpa, is more or less constant. As well as the deposition of starch, this is also the period in which storage protein is deposited, the latter placing a much higher demand on ATP consumption per unit of dry matter deposited (Jenner *et al*., 1991). The effect of inhibiting mitochondrial respiration with antimycin A, on the partitioning of radiolabelled sucrose into starch and protein in isolated endosperms taken at 14 dpa, a time at which both starch and protein are being laid down, was therefore investigated. Figure 4a shows that both the uptake of O_2 and synthesis of ATP were inhibited with increasing concentration of inhibitor. The data presented in Fig. 4b suggest that the impact of restricting the supply of ATP is more marked on starch synthesis than protein synthesis. Whilst there are obvious limitations to the interpretation of these data, they suggest that mechanisms exist within the endosperm to partition the products of respiratory metabolism towards the maintenance of protein synthesis at the expense of starch production. Details of how energy provision is partitioned towards different end-products is, therefore,

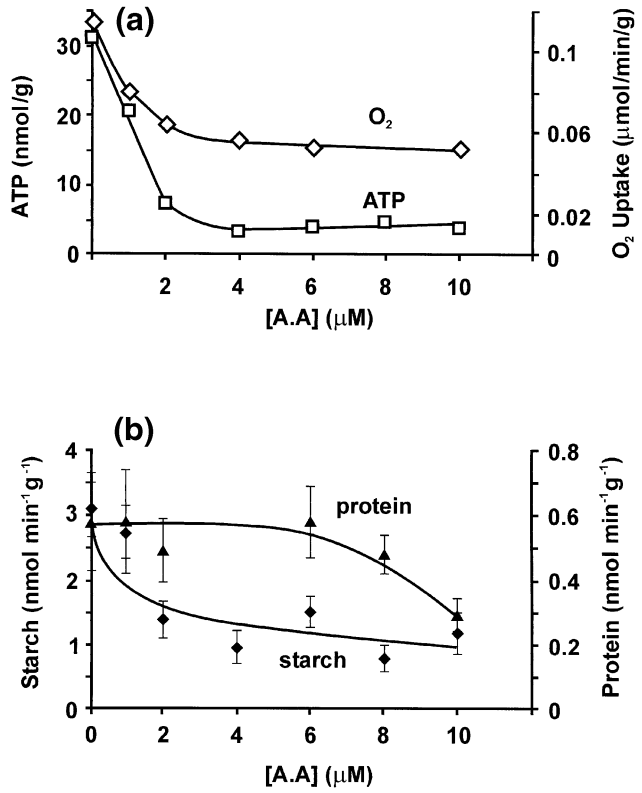

Fig. 4. Effect of antimycin A (A.A) on respiration, ATP, starch and protein synthesis. Endosperm taken at 14 dpa (a) O_2 uptake (open diamonds) and ATP (open squares) content. (b) Incorporation of 20 mM [U-^{14}C] sucrose (37 kBq) into starch (filled diamonds) and protein (filled triangles). Total incubation period in sucrose and antimycin A was 3 h prior to freeze-clamping and tissue extraction (values are mean ±SE of four separate experiments).

a matter for further investigation. Nonetheless, the importance of the adenylate pool in restricting starch synthesis should not be overlooked. Recent studies with potato demonstrated that feeding of exogenous adenine to tuber discs led to elevated concentrations of ADPG and an increase in the rate of starch synthesis (Loef *et al*., 2001).

Conclusions

In summary, therefore, the synthesis of starch in developing cereal endosperm is regulated during development. A key factor which influences both the rate of starch synthesis and the partitioning of carbon between amylose and amylopectin is the production of activated sugar, ADPglucose. In developing endosperm there is strong evidence that this may occur in both the cytosol and amyloplast. Although in barley, maize, and wheat endosperm the activity of amyloplast AGPase varies from 2–30% of the total, there are no data to indicate which predominates *in vivo*. However, the synthesis of an amyloplast ADPglucose transporter during the main period of grain filling adds weight to the argument that the cytosolic production of ADPG is more significant. Finally, the production of ADPG may be constrained by the presence of ADPglucose pyrophosphatase activity, particularly early in development, and the provision of ATP through mitochondrial respiration.

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