

A Cytosolic ADP-Glucose Pyrophosphorylase Is a Feature of Graminaceous Endosperms, But Not of Other Starch-Storing Organs¹

Diane M. Beckles², Alison M. Smith*, and Tom ap Rees³

Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, United Kingdom (D.M.B., T. a.R.); and Department of Applied Genetics, John Innes Centre, Colney Lane, Norwich NR4 7UH, United Kingdom (D.M.B., A.M.S.)

The occurrence of an extra-plastidial isoform of ADP-glucose (Glc) pyrophosphorylase (AGPase) among starch-storing organs was investigated in two ways. First, the possibility that an extra-plastidial isoform arose during the domestication of cereals was studied by comparing the intracellular distribution of enzyme activity and protein in developing endosperm of noncultivated *Hordeum* species with that previously reported for cultivated barley (*Hordeum vulgare*). As in cultivated barley, the AGPase of *H. vulgare* subsp. *spontaneum* and *Hordeum murinum* endosperm is accounted for by a major extra-plastidial and a minor plastidial isoform. Second, the ratio of ADP-Glc to UDP-Glc was used as an indication of the intracellular location of the AGPase activity in a wide range of starch-synthesizing organs. The ratio is expected to be high in organs in which UDP-Glc and ADP-Glc are synthesized primarily in the cytosol, because the reactions catalyzed by AGPase and UDP-Glc pyrophosphorylase will be coupled and close to equilibrium. This study revealed that ADP-Glc contents and the ratio of ADP-Glc to UDP-Glc were higher in developing graminaceous endosperms than in any other starch-storing organs. Taken as a whole the results indicate that an extra-plastidial AGPase is important in ADP-Glc synthesis in graminaceous endosperms, but not in other starch-storing organs.

The intracellular location of ADP-Glc pyrophosphorylase (AGPase) varies from one starch-storing organ to another. For most of the organs for which reliable measurements have been made, the enzyme is exclusively plastidial. These organs include pea embryo and root (Denyer and Smith, 1988; Smith, 1988; Borchert et al., 1993), oilseed rape (*Brassica napus*) embryo (Kang and Rawsthorne, 1994), and potato tuber (Sweetlove et al., 1996; Naeem et al., 1997), and leaves of several species (Okita et al., 1979; Echeverria and Boyer, 1986; Robinson and Preiss, 1987). However, cell fractionation experiments with developing endosperm from cultivated barley (*Hordeum vulgare*) and from maize (*Zea mays*) reveal that 20% or less of the activity is located in the plastid, most of the activity being in an extra-plastidial compartment assumed to be the cytosol (Denyer et al., 1996; Thorbjørnsen et al., 1996). Immunological detection of AGPase proteins in subcellular fractions of maize and barley endosperm, and measurements of metabolites and enzyme activities in fractions of en-

dosperm from maize mutants deficient in an AGPase subunit (*BRITTLE2* mutant) and in a putative plastidial transporter protein (*BRITTLE1* mutant), all support the idea that much of the ADP-Glc for starch synthesis is supplied by a cytosolic rather than a plastidial isoform of AGPase in these organs (Denyer et al., 1996; Shannon et al., 1996, 1998; Thorbjørnsen et al., 1996).

We wished to discover the extent of occurrence of a cytosolic form of AGPase among starch-storing organs. Reliable, quantitative localization experiments have been carried out on relatively few organs (ap Rees, 1995) and it remains possible that cytosolic AGPase is of widespread occurrence. On the other hand, the fact that a cytosolic AGPase has thus far been reported only in cultivated cereals may indicate that it has been selected for during millennia of selective breeding for high grain yields, and is of no significance in wild species.

To examine the possibility that a cytosolic AGPase is confined to the endosperms of domesticated cereals, we have determined the subcellular location of activity and subunit proteins of the enzyme in developing endosperm of barley subsp. *spontaneum* (referred to as *Hordeum spontaneum*), a putative progenitor of modern barley, which may have been subjected to some selection by early farmers, and *Hordeum murinum*, a wild species. We present data that establish that most of the AGPase is extra-plastidial in these endosperms.

We considered it impractical to attempt to discover the general importance of extra-plastidial

¹ This work was supported by a Competitive Strategic Grant from the Biotechnology and Biological Sciences Research Council (UK) at the John Innes Centre. D.M.B. was supported by a studentship from the Commonwealth Scholarship Commission.

² Present address: DuPont Agricultural Products, Experimental Station, P.O. Box 80402, Wilmington, DE 19880-0402.

³ Deceased. This work was started in Tom ap Rees's laboratory prior to his death in 1996.

* Corresponding author; e-mail alison.smith@bbsrc.ac.uk; fax 44-1603-450045.

AGPase among higher plants through cellular fractionation experiments. Instead we have assessed whether starch-storing organs from a wide range of species are likely to have significant extra-plastidial activity by measuring the amounts of the sugar nucleotides ADP-Glc and UDP-Glc during the period of starch synthesis. The rationale for this approach is as follows. UDP-Glc is made exclusively in the cytosol and its level is determined primarily by two reactions catalyzed by Suc synthase and UDP-Glc pyrophosphorylase, which are close to equilibrium in vivo (ap Rees et al., 1984, 1988; Edwards and ap Rees, 1986; Geigenberger and Stitt, 1993). If AGPase is present in the cytosol, then the level of ADP-Glc in this compartment might be expected to approach that of UDP-Glc because the two pyrophosphorylytic reactions will be coupled by common substrates (Kleczkowski, 1994). In contrast, the level of ADP-Glc in tissues with exclusively plastidial AGPase activity is determined by two physiologically irreversible reactions and is highly unlikely to be directly related to the level of UDP-Glc. In the plastid, the pyrophosphate (PPi) produced by AGPase is believed to be cleaved by plastidial alkaline inorganic pyrophosphatase, rendering the synthesis of ADP-Glc irreversible (Gross and ap Rees, 1986). Further, the ADP-Glc in the plastid is immediately available to the starch synthases, which catalyze a second irreversible reaction. These considerations suggest that ADP-Glc and UDP-Glc levels may be similar in tissues with cytosolic AGPase, but could be very different in tissues with exclusively plastidial AGPase.

RESULTS

AGPase Activity Is Associated with Plastidial and Extra-Plastidial Fractions of the Endosperm of Barley Species

Pellets enriched in plastids were prepared by low-speed centrifugation from homogenates of developing endosperm of *H. spontaneum* and *H. murinum*. The extent of enrichment of the pellet with plastids was assessed by comparison of activities of the exclusively plastidial enzymes soluble starch synthase and alkaline pyrophosphatase (referred to as plastidial marker enzymes) and the exclusively cytosolic enzymes PPi, Fru-6-P 1-phosphotransferase (PFK), and alcohol dehydrogenase (ADH; referred to as cytosolic marker enzymes) in the homogenate and the pellet. Losses of activity of these enzymes during cellular fractionation were minimal (Table I, Pellet + Supernatant values). For *H. spontaneum*, the pellet contained 15% to 16% of the total activity of the plastidial marker enzymes and less than 2% of the total activity of the cytosolic marker enzymes. For *H. murinum* these values were 10% to 17% for plastidial marker enzymes and less than 1% for cytosolic marker enzymes (Table I).

To determine the location of AGPase, the percentage of the total activity of this enzyme recovered in the pellet was compared with the values for plastidial and cytosolic marker enzymes (Table I). For both species the value for AGPase was statistically significantly lower than that of the plastidial marker enzymes, but higher than that of the cytosolic marker enzymes (Student's *t* test, $P < 0.01$). This strongly suggests that some of the AGPase activity is plastidial, but much is extra-plastidial. The percentage of activity that was extra-plastidial was estimated to be 75% or higher, based on values from four to six experiments (Table I).

Although yields of plastids from both species were reasonable, the enrichment in plastidial relative to cytosolic markers of the pellets (about 10-fold) was not as great as that obtained in previous AGPase localization experiments with maize and barley endosperm (enrichments of 15- and 29-fold, respectively; Denyer et al., 1996; Thorbjørnsen et al., 1996). To discover whether the relatively low levels of enrichment affected the apparent distribution of AGPase activity, the distribution of this activity was investigated in pellets prepared from a single homogenate and deliberately contaminated to different degrees with cytosol. Two features of the results allow us to conclude that the relatively low levels of enrichment do not affect the interpretation of our localization experiments. First, when the activity of AGPase and that of a cytosolic marker enzyme are expressed as fractions of the activity of a plastidial marker enzyme, the ratio between these values is the same for all of the pellets (Fig. 1). This means that the estimated percentage of AGPase activity that is apparently extra-plastidial is the same regardless of the degree of cytosolic contamination of the pellet. Second, regression analysis of the data in Figure 1 gives values for the percentage of AGPase activity that is extra-plastidial of 76% for *H. spontaneum* and 81% for *H. murinum*: these values are close to those obtained from the independent fractionation experiments presented in Table I.

As a final check on the location of AGPase, we compared activity in assays of homogenates in which plastids were intact or ruptured to obtain a percentage latency value (Table II). As expected, activity of the cytosolic marker enzyme was similar whether or not the plastids were ruptured (latencies of 12% and 5% for *H. murinum* and *H. spontaneum*, respectively), and activity of the plastidial marker enzyme was much higher when plastids were ruptured than when they were intact (latencies of 82% and 51% for *H. murinum* and *H. spontaneum*, respectively). Latencies for AGPase were higher than those of the cytosolic marker enzyme, but much lower than those of the plastidial marker enzyme (values of 38% and 14% for *H. murinum* and *H. spontaneum*, respectively), consistent with the idea that some of the AGPase activity is plastidial, but most is extra-plastidial.

Table 1. Activities of cytosolic and plastidial marker enzymes and of AGPase in fractions of endosperm of *H. spontaneum* and *H. murinum*

For measurements of total activity in endosperm, extracts were prepared without osmoticum by a method ensuring complete breakage of cells. For preparation of homogenate, pellet, and supernatant fractions, endosperms were first plasmolyzed in an osmoticum, then chopped with razor blades in a medium containing osmoticum. Debris was removed by filtration, and the resulting homogenate was subjected to low-speed centrifugation to yield pellet and supernatant, and pellet fractions were treated with Triton X-100 or extruded through a fine-bore needle to rupture organelles prior to assay. To estimate the percentage of the total AGPase activity that is extra plastidial, it was assumed that the activity of AGPase in the pellet fraction is composed of a plastidic and a cytosolic component, and that these sediment to the same extent as the plastidial and cytosolic marker enzymes. The following equation was then applied:

$$\text{Percentage of activity that is extra plastidial} = \frac{\% \text{ of PMA in the pellet} - \% \text{ of total AGPase activity in pellet}}{\% \text{ of PMA in the pellet} - \% \text{ of CMA in the pellet}}$$

where PMA is activity of the plastid marker enzyme (either alkaline pyrophosphatase or soluble starch synthase) and CMA is the activity of the cytosolic marker enzyme (mean of the alcohol dehydrogenase and PFP values used in the calculations). All values are means \pm SE of measurements made on the number of separately prepared homogenates shown in parentheses, or for values of total activity in endosperm, measurements from three separate extracts. PFP, pyrophosphate, Fru-6-P 1-phosphotransferase.

Species	Enzyme (Cellular Location)	Total Activity in Endosperm	Activity as a Percentage of That in the Original Homogenate		Estimated Percentage of Total Activity Outside Plastid	
			Pellet	Pellet + supernatant	From alkaline pyrophosphatase	From soluble starch synthase
		$\mu\text{mol min}^{-1}\text{g}^{-1}$ fresh wt				
<i>H. spontaneum</i>	Alkaline pyrophosphatase (plastid)	1.3 ± 0.1	16.0 ± 2.6	113 ± 13 (4)	–	–
	Soluble starch synthase (plastid)	0.08 ± 0.002	15.0 ± 4.4	108 ± 12 (4)	–	–
	Alcohol dehydrogenase (cytosol)	1.4 ± 0.03	1.3 ± 0.6	111 ± 15 (4)	–	–
	PFP (cytosol)	8.4 ± 1.3	1.6 ± 0.7	112 ± 12 (4)	–	–
	AGPase	2.7 ± 0.2	5.1 ± 1.3	112 ± 11 (4)	81	75
<i>H. murinum</i>	Alkaline pyrophosphatase (plastid)	8.0 ± 0.4	17.0 ± 1.5	93 ± 4 (9)	–	–
	Soluble starch synthase (plastid)	1.0 ± 0.01	10.0 ± 1.8	103 ± 3 (5)	–	–
	Alcohol dehydrogenase (cytosol)	1.4 ± 0.4	0.9 ± 0.9	90 ± 6 (7)	–	–
	PFP (cytosol)	8.0 ± 0.02	0.2 ± 0.2	95 ± 3 (9)	–	–
	AGPase	5.7 ± 0.6	2.9 ± 0.4	79 ± 5 (7)	89	75

Different AGPase Proteins Are Present in Plastidial and Extra-Plastidial Fractions of Endosperm

In endosperms of a modern, cultivated barley and maize, the plastidial and extra-plastidial forms of the small subunit of AGPase are distinct proteins of different molecular masses (Denyer et al., 1996; Thorbjørnsen et al., 1996). We checked whether this was also the case for endosperms of *H. spontaneum* and *H. murinum*. Samples of homogenates and of pellet and supernatant fractions derived from them were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. To minimize proteolytic degradation of AGPase proteins, the extraction medium contained the protease inhibitors phenylmethylsulfonyl fluoride and chymostatin (Plaxton and Preiss, 1987; Kleczkowski et al., 1993). Blots were developed with antiserum against the major form of the small subunit of AGPase from maize endosperm (the BRITTLE2 gene product; Giroux and Hannah, 1994). This antiserum recognizes plastidial and extra-plastidial forms of the small subunit in maize and barley endosperms (Denyer et al., 1996; Thorbjørnsen et al., 1996). When lanes

were loaded so that each contained the same activity of the plastidial marker enzyme alkaline pyrophosphatase, the antiserum recognized a major protein of approximately 54 kD, and occasionally a minor protein of 51 kD, in homogenate and supernatant fractions. However, it recognized little or no protein in pellet fractions (Fig. 2A). This indicates that the 54-kD AGPase subunit is not plastidial. When lanes were loaded so that each contained an equal activity of the cytosolic marker enzyme ADH, there were approximately equal amounts of the 54-kD protein in pellet and homogenate lanes, but the pellet lane was strongly enriched in the 51-kD protein relative to the homogenate lane (Fig. 2B). This provides further evidence that the 54-kD protein is extra-plastidial, and suggests that the 51-kD protein is plastidial. Taken as a whole, the results are consistent with the idea that there are two distinct forms of the small subunit in *H. spontaneum* and *H. murinum* endosperms: a form of lower molecular mass that is enriched in or confined to the plastid, and a form of higher molecular mass that is enriched in or confined to the cytosol. The

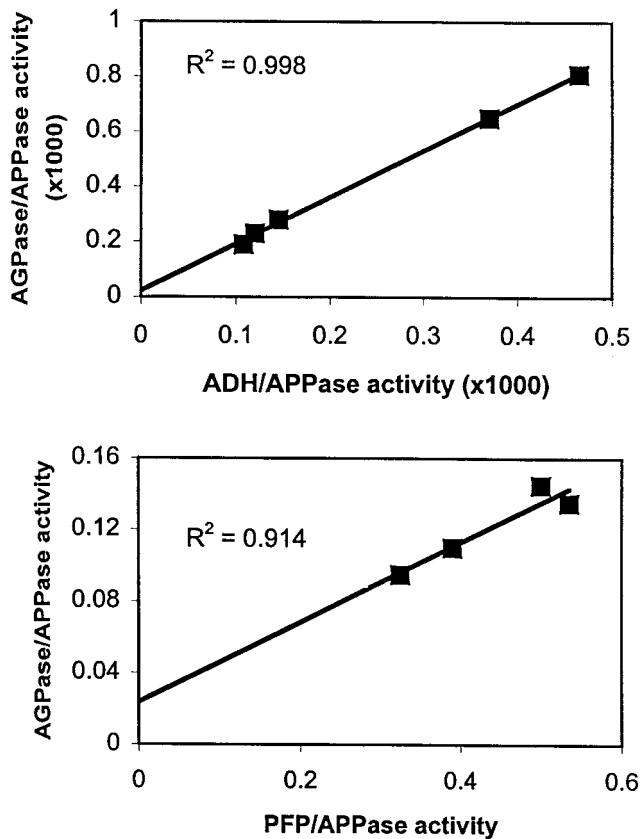


Figure 1. Subcellular distribution of AGPase activity in barley endosperm determined by the ratio method. Five samples of amyloplast-enriched pellet, each contaminated to a different extent with cytosol, were obtained from a single homogenate of endosperm of *H. murinum* or *H. spontaneum* as follows. A sample of the homogenate was taken for assays as described below, then the remainder was divided into four or five samples, each of which was centrifuged identically to produce pellets and supernatant fractions. The supernatant fractions were pooled together. The pellets were resuspended in 1 mL of material that consisted entirely of the supernatant fraction, or of a mixture of the supernatant fraction and AIM, or entirely of AIM. After rupture of the plastids and centrifugation, each of these four or five samples was assayed for a cytosolic marker enzyme, ADH or PFP, for the plastidial marker enzyme alkaline pyrophosphatase (APPase), and for AGPase. The distribution of AGPase activity was determined from the slopes of the plots of (AGPase activity)/(plastid marker enzyme activity) versus (cytosolic marker enzyme activity)/(plastid marker enzyme activity) for these samples (as shown), according to the following assumption: Total AGPase activity = $C_1 + C_2(\text{CMA/PMA})$, where $C_1 = \text{pAGPase/PMA}$ and $C_2 = \text{cAGPase/CMA}$. PMA, Plastid marker enzyme activity in the unfractionated homogenate; CMA, cytosolic marker enzyme activity in the unfractionated homogenate; pAGPase, plastidial AGPase activity; cAGPase, cytosolic AGPase activity. Top graph, *H. spontaneum*. Bottom graph, *H. murinum*. Data from the graphs were as follows: *H. spontaneum*, $y = 1.73x + 0.143$ (hence, $C_1 = 0.143$ and $C_2 = 1.73$), CMA/PMA = 0.097; and *H. murinum*, $y = 0.220x + 0.025$ (hence, $C_1 = 0.025$ and $C_2 = 0.220$), CMA/PMA = 0.560.

presence of the putative cytosolic form as well as the plastidial form in the pellet is attributable to the contamination of this fraction by cytosol (see Table I).

The Ratio of ADP-Glc to UDP-Glc Is Higher in Cereal Endosperms than in Other Starch-Storing Organs

Amounts of ADP-Glc and UDP-Glc were measured in starch-storing organs from a wide range of plants, including roots, tubers, embryos, and endosperms from monocotyledonous and dicotyledonous species. The following is evidence that our measurements reflect closely the amounts in the tissues. First, the time between harvesting the tissue and freezing in liquid nitrogen was minimal (typically 20–360 s). Second, to ensure uniform freezing, bulky tissue was freeze-clamped (ap Rees, 1974). Where tissue was routinely frozen rather than freeze-clamped, results were validated by comparing the levels of ADP-Glc and UDP-Glc in duplicate samples of tissue, one of which had been frozen and the other freeze-clamped. Third, our measurements of ADP-Glc and UDP-Glc by HPLC were reproducible and reliable. The elution times of the two compounds differed by at least 3 min. In addition, of 12 other nucleotides tested, only UDP-GlcNac with a retention time (Rt) of 32.88 min and UDP-*N*-acetylgalactosamine (Rt = 33.70 min) eluted close to standard ADP-Glc (Rt = 31.33 min) and UDP-Glc (Rt = 34.74 min), and these eluted as discrete peaks. These acetylated nucleotides are in any case either of very low abundance or absent from plant tissues. For extracts of broad bean (*Vicia faba*) embryo and wheat and barley endosperm, the ADP-Glc that eluted from the column was hydrolyzed and then assayed enzymatically for Glc and ADP. The amounts of these compounds were within 16% of the amount of ADP-Glc measured by HPLC (results not shown). This indicates that the HPLC peak was predominantly or solely ADP-Glc. Fourth, we checked the reliability of the extraction and assay methods by recovery experiments. For each tissue, duplicate samples were extracted similarly, except that ADP-Glc and UDP-Glc were added to one sample before extraction in amounts similar to those expected from previous measurements to be present in the sample. The differences between the duplicate samples in the amounts of each compound measured are expressed as percentages of the amounts added. The recoveries were for the most part within 20% of those expected, indicating that there was no serious loss of either compound during analysis. Recoveries just outside this range may simply reflect the difficulty of sampling heterogeneous organs. We repeatedly obtained low recoveries of ADP-Glc for cassava root (*Manihot esculenta*; 20%–28%; data not shown), which underscores the importance of this check. The recovery of UDP-Glc for taro corm (*Colocasia esculenta*) was high (145%). However, overestimation of UDP-Glc by this amount would not affect our interpretation of the ratio of ADP-Glc to UDP-Glc in this tissue (see below). Table III contains data only for those organs and stages of development for which recoveries of ADP-Glc were within 23% of those expected.

Table II. Latencies of enzyme activities in plastid-enriched pellets from *Hordeum endosperms*

Duplicate samples of homogenate were prepared as described for Table I. Plastids in one of the two samples were broken by repeated extrusion through a fine-bore needle. The activities of AGPase and the cytosolic marker enzyme in the two samples were assayed in the presence of 0.6 M sorbitol. The activity of the plastidial marker enzyme alkaline pyrophosphatase was assayed in the presence of 0.6 M Suc because sorbitol has been shown to inhibit this activity (Tetlow et al., 1993). The activity in the ruptured sample minus that in the intact sample is expressed as a percentage of that in the ruptured sample, and this is presented as the latency value. In each experiment, each enzyme in the two samples was assayed three times. Values are means \pm se of measurements from three separate experiments.

Species	Enzyme	Activity		Latency
		Intact	Ruptured	
		<i>nmol min⁻¹ mL⁻¹</i>		%
<i>H. spontaneum</i>	Alkaline pyrophosphatase	451 \pm 9	896 \pm 380	51 \pm 6
	Alcohol dehydrogenase	410 \pm 19	431 \pm 13	5 \pm 3
	AGPase	190 \pm 4	220 \pm 14	14 \pm 3
<i>H. murinum</i>	Alkaline pyrophosphatase	120 \pm 1	690 \pm 1	82 \pm 5
	Alcohol dehydrogenase	129 \pm 1	131 \pm 1	12 \pm 2
	AGPase	190 \pm 1	303 \pm 1	38 \pm 14

To make valid comparisons of the ratio of ADP-Glc to UDP-Glc between different tissues, it was important to ensure that all tissues were actively synthesizing starch at the time of sampling. For organs where relatively little information is available about starch synthesis (horse chestnut, sweet pea, broad bean and oak embryos, yam [*Dioscorea bulbifera*] tuber, tomato fruit, and rice, millet [*Eleusine coracana*], darnel [*Lolium temulentum*], and wild oat [*Avena fatua*] endosperm) we assayed starch content and starch synthase activity around the developmental point at which the tissue was sampled for sugar nucleotide measurements (data not shown). These measurements confirmed that sampling was carried out at a point when starch content was increasing. Activities of starch synthase were in the range 0.03 to 0.2 $\mu\text{mol min}^{-1} \text{g}^{-1}$ fresh weight; values that are comparable with those in well-studied starch-storing organs such as pea embryo (Smith et al., 1989) and potato tuber (Edwards et al., 1995).

The measurements of ADP-Glc and UDP-Glc (Table III) show that the amount of ADP-Glc per gram of fresh weight and the ratio of ADP-Glc to UDP-Glc were higher in all of the graminaceous endosperms than in any of the other organs examined. Ratios ranged from 0.3 to 0.64 for endosperms, and from 0.01 to 0.18 for all other organs.

DISCUSSION

AGPase Is Mainly Extra Plastidial in Endosperms of Cultivated and Wild Barley Species

Three lines of evidence from cell-fractionation experiments lead us to conclude that most of the AGPase activity in the endosperms of *H. spontaneum* and *H. murinum* is extra-plastidial, and there are distinct plastidial and extra-plastidial isoforms of the enzyme. First, the percentage of the total activity of AGPase that sediments with the plastid fraction is

greater than that of cytosolic marker enzymes, but considerably less than that of plastidial marker enzymes. This result is highly reproducible and is not affected by the relatively low enrichment of plastids in the pellet fractions in these experiments. Second, AGPase activity in the plastid fraction exhibits a latency value higher than that of the cytosolic marker, but considerably lower than that of the plastidial marker. This result is expected of an enzyme with a major extra-plastidial and a minor plastidial component. Third, immunoblot analysis reveals two forms of the small subunit of AGPase in the endosperm, of different molecular masses. The smaller of the two forms is enriched in the plastid fraction, but the larger is not. This implies that the small subunit responsible for plastidial activity is a different protein from that responsible for extra-plastidial activity.

These results for ancestral and wild barleys are very similar to those we obtained previously for a cultivated barley. Our study of developing endosperm of barley cv Bomi revealed that about 85% of the activity was extra-plastidial and that the subunits of plastidial and extra-plastidial forms of the enzyme are of different molecular masses (Thorbjørnsen et al., 1996). We conclude that the existence of the extra-plastidial form of AGPase in cultivated barley is not the result of selective breeding for high starch content. The occurrence of plastidial and extra-plastidial forms of the enzyme appears to be a feature of cultivated and wild species of barley.

A Cytosolic AGPase May Be Present Only in Graminaceous Endosperm

Our measurements of ADP-Glc and UDP-Glc (Table III) show that the amount of ADP-Glc per gram of fresh weight and the ratio of ADP-Glc to UDP-Glc were higher in all of the graminaceous endosperms

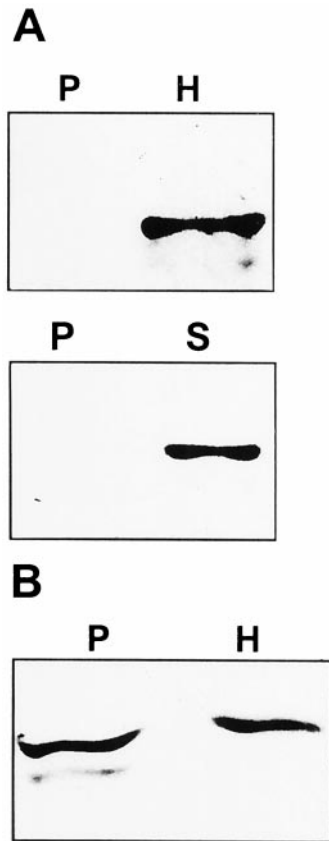


Figure 2. Detection of AGPase small subunits by immunoblotting of fractions from amyloplast preparations. Homogenate, pellet, and supernatant fractions were prepared from endosperms of *H. spontaneum* and *H. murinum* as described in "Materials and Methods." They were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. Blots were developed with antiserum against the major form of the small subunit of AGPase from maize endosperm (the BRITTLE2 gene product), at a dilution of 1/15,000. To obtain the pellet fractions shown in B, samples of pellets were concentrated by freeze drying. Blots were repeated on several amyloplast preparations from each species. Typical, representative examples are shown. H, Homogenate; P, pellet; S, supernatant. A, Lanes all contain the same activity of the plastidial marker enzyme alkaline pyrophosphatase. Top, *H. spontaneum*. Bottom, *H. murinum*. B, Lanes contain the same activity of the cytosolic marker enzyme ADH. Samples are from a single plastid preparation from *H. spontaneum*.

than in any of the other organs examined. The ratios for endosperms (0.3–0.64) are comparable with those previously reported for the developing endosperm of maize (0.35; Shannon et al., 1996) and whole barley grain (0.21; Tyynelä et al., 1995). Of the ratios for other organs the highest value was for broad bean embryo (0.18). This value is comparable with that in pea embryos [0.16, estimated from Edwards and ap Rees (1986) and Edwards et al. (1988)]. All other organs had ratios in the range 0.01 to 0.04. Values reported previously for potato tuber (0.01–0.02; Geigenberger et al., 1994; Sweetlove et al., 1996) also fall within this range.

These results are consistent with our expectation that amounts of ADP-Glc and UDP-Glc will be similar

in tissues with cytosolic AGPase, but may be different in tissues with exclusively plastidial AGPase. The ADP-Glc to UDP-Glc ratio is higher in the organs in which the existence of a cytosolic AGPase has been established by cell fractionation experiments (species of barley and maize) than in any of the organs in which AGPase has been shown to be essentially confined to the plastid (for example pea embryo, oilseed rape embryo, and potato tuber). We are therefore confident that the ratio of ADP-Glc to UDP-Glc may be used to indicate whether extra-plastidial AGPase is present in starch-storing tissues. We accordingly conclude that a cytosolic AGPase makes a major contribution to ADP-Glc synthesis in graminaceous endosperms, but probably does not do so in a wide range of other non-photosynthetic starch-storing organs.

Our conclusions about the location of AGPase are not consistent with those drawn for maize endosperm (Miller and Chourey, 1995; Brangeon et al., 1997) and tomato fruit (Chen et al., 1998) from immunolabeling experiments using antisera against AGPase. For maize endosperm, labeling was seen only within the plastid during much of development, and the authors cast doubt upon the idea that most of the activity is extra-plastidial. For tomato, labeling was seen in the cytosol as well as in the plastid, and AGPase activity was proposed to be present in both compartments. We point out that immunogold labeling reveals the distribution of antigenic protein in a fixed and embedded section, which may not necessarily reflect the distribution of AGPase activity in the living cell. A study of the location of AGPase activity in cells of tomato fruit is in progress.

Possible Function of a Cytosolic AGPase

The advantage conferred by the possession of cytosolic AGPase remains enigmatic. However, we speculate that it may facilitate the partitioning of large amounts of carbon from Suc into starch when there is a plentiful supply of Suc in the endosperm. In tissues in which AGPase is exclusively plastidial, the pathway from Suc to starch involves the import of hexose phosphate and ATP into the plastid. The resulting plastidial pools of these metabolites are used not only for starch synthesis, but also, for example, for fatty acid synthesis, amino acid synthesis, and the oxidative pentose phosphate pathway. In contrast, the possession of a cytosolic as well as a plastidial AGPase allows the direct commitment of carbon from Suc into the pathway of starch synthesis without the involvement of the plastidial hexose phosphate and ATP pools. The extent of this commitment may be dependent upon the concentration of Suc in the cytosol. When the Suc concentration is high, cytosolic ADP-Glc concentrations will also be high because the enzymes that convert Suc to ADP-Glc are close to equilibrium. When Suc concentration is low,

Table III. ADP-Glc and UDP-Glc contents of starch-storing organs

Organs were harvested at the developmental stage indicated, and samples of between 8 and 800 mg (depending on the organ) were immediately frozen, either directly in liquid nitrogen or by freeze clamping. ADP-Glc and UDP-Glc were measured by HPLC analysis of neutralized perchloric acid extracts, performed on a Partisil-10-SAX column. For recovery experiments, samples were divided in half and frozen separately. An amount of pure ADP-Glc or UDP-Glc equal to that estimated to be in the tissue was added to one-half with the perchloric acid. The recovery value is the difference in ADP-Glc or UDP-Glc content of the two halves, expressed as a percentage of the amount added. Values are means \pm SE of measurements made on the number of separate extracts shown in parentheses in the final column, except where recovery experiments were done on a different number of samples (shown in parentheses in the "Recovery" columns). Where samples consisted of part of an organ, each extract was made from a separate organ. DPP, Days post-pollination; DPA, days post-anthesis.

Organ	Developmental Stage (DPA/DPP or fresh wt)	ADP-Glc	Recovery of	UDP-Glc Content	Recovery of	Ratio of
		Content	ADP-Glc	UDP-Glc	UDP-Glc	ADP-Glc to UDP-Glc
		<i>nmol g⁻¹ fresh wt</i>	%	<i>nmol g⁻¹ fresh wt</i>	%	
Cereal endosperm						
Barley	10 DPA	113 \pm 6	74 \pm 9	258 \pm 12	93 \pm 5	0.44 (6)
	30 DPA	108 \pm 9	114 \pm 9 (4)	233 \pm 13	124 \pm 12 (4)	0.46 (6)
Wheat	10 DPA	168 \pm 3	99 \pm 4 (4)	425 \pm 20	89 \pm 4	0.40 (6)
	30 DPA	156 \pm 5	86 \pm 9	301 \pm 18	85 \pm 11	0.46 (6)
Oat	10 DPA	126 \pm 5	91 \pm 8	393 \pm 18	130 \pm 4	0.32 (6)
Maize	15–17 DPA	231 \pm 19	99 \pm 11	556 \pm 57	96 \pm 11	0.42 (5)
	32–35 DPA	217 \pm 13	103 \pm 9	340 \pm 9	116 \pm 10	0.64 (6)
Millet	4 mg	57 \pm 3	96 \pm 6	272 \pm 7	107 \pm 5	0.38 (6)
Wild oat	25 DPA	100 \pm 6	92 \pm 6	262 \pm 22	92 \pm 5	0.38 (6)
Darnel	10 DPA	75 \pm 5	103 \pm 7	134 \pm 6	96 \pm 3	0.54 (6)
	20 DPA	52 \pm 23	79 \pm 12 (4)	152 \pm 24	87 \pm 27 (4)	0.34 (6)
<i>H. spontaneum</i>	16 DPA	116 \pm 9	98 \pm 8	213 \pm 18	101 \pm 10	0.55 (6)
<i>H. murinum</i>	10 DPA	46 \pm 4	83 \pm 8 (4)	151 \pm 10	100 \pm 18 (4)	0.30 (6)
Other organs						
Oilseed rape embryo	2 mg	3.1 \pm 0.3	77 \pm 4	296 \pm 10	110 \pm 9	0.01 (6)
Tomato fruit pericarp	9 DPP	1.3 \pm 0.6	95 \pm 9	127 \pm 17	86 \pm 9	0.01 (6)
Tomato fruit columella plus placenta	12 DPP	1.9 \pm 0.5	92 \pm 2	86 \pm 5	70 \pm 3	0.02 (6)
	16 DPP	2.4 \pm 0.2	104 \pm 13	134 \pm 18	89 \pm 9	0.02 (6)
Horse chestnut embryo	26 \pm 4 g	2.3 \pm 1.3	115 \pm 10	166 \pm 1	117 \pm 11 (5)	0.01 (6)
Oak embryo	1.4 \pm 0.2 g	4.3 \pm 2.0	92 \pm 9	102 \pm 12	92 \pm 6	0.04 (6)
Taro corm	2.5 \pm 1.5 g	2.0 \pm 0.5	103 \pm 11 (4)	141 \pm 59	145 \pm 18 (4)	0.01 (4)
Yam tuber	10 \pm 4 g	2.4 \pm 1.0	90 \pm 10	80 \pm 6	90 \pm 10	0.03 (12)
Sweet pea embryo	9 \pm 2 mg	9.2 \pm 2.1	83 \pm 12 (4)	344 \pm 46	112 \pm 16 (4)	0.03 (6)
Broad bean embryo	2.3 \pm 0.4 g	53 \pm 3	83 \pm 7 (4)	301 \pm 16	80 \pm 7 (4)	0.18 (5)

most of the ADP-Glc for starch synthesis will be provided via the import of hexose phosphates into the plastid. This mechanism thus ensures that carbon is available for processes other than starch synthesis when Suc supply is limited, but allows carbon from Suc to be committed directly to starch when Suc is plentiful.

MATERIALS AND METHODS

Plant Material

Wheat (*Triticum aestivum* cv Troy), barley (*Hordeum vulgare* cv Halcyon, *H. spontaneum*, and *H. murinum*), oat (*Avena sativa* cv Piper), wild oat (*Avena fatua*), darnel (*Lolium temulentum* L.), millet (*Eleusine coracana* var KNE 626), maize (*Zea mays* var LG5080), tomato (*Lycopersicon esculentum* cv MoneyMaker), and broad bean (*Vicia faba* cv Sutton Dwarf) were grown in soil-based compost in a greenhouse with supplementary lighting in winter and a minimum temperature of 18°C to 22°C (millet and maize) or 12°C to 15°C (other species). Plants of barley, *Hordeum spontaneum*, wheat, and oats were held at 4°C for 6 weeks after sowing

prior to transfer to the greenhouse. Yam (*Dioscorea bulbifera* cv Dahlbergia), taro (*Colocasia esculenta* cv Bali), and cassava (*Manihot esculenta* var CMC-40) were propagated vegetatively, and grown under conditions similar to those for other species with a minimum temperature of 22°C to 25°C. Oilseed rape (*Brassica napus* cv Topas) was grown according to Kang and Rawsthorne (1994). All other plant material was harvested directly from plants growing in the wild or in gardens in Cambridge or Norwich (UK) in summer.

Preparation of Amyloplasts

The method was modified from that of Tetlow et al. (1993) and Thorbjørnsen et al. (1996). Endosperms of up to 11 d post-anthesis (DPA) from *Hordeum murinum* (0.8–1 g in total) or up to 13 DPA from *H. spontaneum* (0.3–0.5 g in total) were gently squeezed into a medium containing 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 7.5], 0.5 M sorbitol, and washed twice in this medium before plasmolyzing for 30 to 60 min in 0.8 M sorbitol, 1 mM EDTA, 1 mM KCl, and 2 mM MgCl₂. Endosperms were then chopped in amyloplast isolation medium (AIM) containing

N-2,3-dihydroxypropylacetamido-2,4,6-triiodo-*N,N'*-bis-(2, 3-dihydroxypropyl) isophthalamide (Nycodenz) at 2 g L⁻¹ for *H. spontaneum* and 0.5 g L⁻¹ for *H. murinum*. Where fractions were to be used for enzyme assays, AIM consisted of 50 mM HEPES (pH 7.5), 0.5 M sorbitol, 1 mM EDTA, 1 mM KCl, 2 mM MgCl₂, 100 mL L⁻¹ (v/v) ethanediol, 1 mM dithiothreitol, and 20 g L⁻¹ of bovine serum albumin. The homogenate (approximately 6 mL) was filtered through two layers of Miracloth and a sample was removed for enzyme assays. The remainder was centrifuged in a swing-out rotor at 50g for 8 min (*H. spontaneum*) or 10 min (*H. murinum*) to produce a supernatant and an amyloplast-enriched pellet fraction. The pellets were resuspended in 2 to 3 mL of AIM for enzyme assays. Any intact amyloplasts in the homogenate, pellet, and supernatant fractions were ruptured prior to assay, either mechanically by vortex-mixing or passage through a fine-bore hypodermic needle, or by addition of 0.1 mL L⁻¹ Triton X-100.

Where fractions were to be used for immunoblot analysis the procedure was the same except that AIM contained 1.5 mM phenylmethylsulfonylfluoride, 0.5 mM chymostatin, and no bovine serum albumin.

Enzyme Assays

All assays were conducted at 25°C.

ADP-Glc Pyrophosphorylase

Spectrophotometric assay containing in 1 mL: 75 mM HEPES (pH 7.9), 5 mM MgCl₂, 1 mM ADP-Glc, 1.5 mM Na PPi, 1.3 mM NAD, 4 units phosphoglucomutase, 10 units of Glc-6-P dehydrogenase (from *Leuconostoc mesenteroides*), and 50 μL of extract.

Soluble Starch Synthase

Radiometric assay containing in 100 μL: 150 mM Bicine [N,N'-bis(2-hydroxyethylglycine); pH 8.4], 0.48 mg of potato amylopectin, 1.4 mM ADP-[U-¹⁴C]Glc at 3 GBq mol⁻¹, and 10 μL of extract. After a 15-min incubation, assays were processed by the resin method described by Jenner et al. (1994). Starch storing-organs were extracted for starch synthase assays as described in Sweetlove et al. (1996).

Alkaline Pyrophosphatase

The assay was performed according to Gross and ap Rees (1986). The incubation contained in 200 μL: 50 mM Bicine (pH 8.9), 20 mM MgCl₂, 1.25 mM Na PPi, and 25 μL of extract. Phosphate was measured with an acid molybdate reagent.

PPF

Spectrophotometric assay containing in 1 mL: 65 mM HEPES (pH 7.9), 5 mM MgCl₂, 5 mM Fru-6-P, 0.2 mM Fru-2,6-bisP, 5 mM KCl, 0.2 mM NADH, 1 unit of aldolase, 1.3 units of glycerol-3-P dehydrogenase, 10 units of triose-P dehydrogenase, and 25 μL of extract.

ADH

Spectrophotometric assay containing in 1 mL: 80 mM glycyl-Gly (pH 8.9), 1.3 mM NAD, 100 mM ethanol, and 25 μL of extract.

SDS-PAGE and Immunoblotting

Samples were diluted 1:1 with double-strength gel sample buffer (Laemmli, 1970; except that mercaptoethanol was replaced with 70 mM dithiothreitol) and boiled 2 min prior to loading onto 7.5% (w/v) SDS-polyacrylamide gels. Gels were stained with Brilliant Blue R. Immunoblotting was according to Bhattacharyya et al. (1990).

Sampling of Tissue for Extraction of ADP-Glc and UDP-Glc

Duplicate samples of all tissues were frozen or freeze-clamped and stored in liquid nitrogen after removal from the plant. The time between removal of the tissue from the plant and freezing or freeze-clamping was less than 20 s for oilseed rape embryos, less than 6 min for horse-chestnut and oak embryos, yam tubers, and taro corms, and 1 to 2 min or less for all other organs.

Cereal endosperm was isolated from grain by squeezing or rapid dissection, and frozen in liquid nitrogen. Samples varied from 25 to 150 mg fresh weight. All embryos except those of oilseed rape were freeze-clamped. Samples consisted of one embryo or part of an embryo and ranged from approximately 30 mg (sweet pea) to about 750 mg (horse chestnut). For oilseed rape, samples consisted of about 20 embryos each of about 2.5 mg. Embryos were dissected from testas with a needle and frozen. For yam tuber and taro corm, pieces of the inner tissue of 0.5 to 1.5 g were removed with a cork borer and freeze-clamped. For tomato fruit, the locular tissue was discarded and the remainder was divided into the pericarp tissue and columella plus placental tissue. Samples of 1 to 2 g of each were finely sliced then frozen.

Extraction and Assay of ADP-Glc and UDP-Glc

ADP-Glc and UDP-Glc were extracted using perchloric acid and assayed by HPLC analysis of neutralized extracts on a Partisil-10-SAX column (Hichrom Ltd., Reading, UK) as described by ap Rees et al. (1984) with the following modifications. The elution program was: 0 to 15 min, buffer A at 0.8 mL min⁻¹; 15 to 43 min 70% (v/v) buffer A and 30% (v/v) buffer B at 1.6 mL min⁻¹; 43 to 50 min 100% (v/v) buffer B at 1.6 mL min⁻¹; and 50 to 62 min 100% (v/v) buffer A at 1.6 mL min⁻¹. Buffer A was 10 mM NH₄H₂PO₄ (pH 3.0) and buffer B was 450 mM NH₄H₂PO₄ (pH 4.3). The column was washed with methanol after every six chromatograms. Each compound was identified by comparison of retention times of, and co-chromatography with, pure samples of each compound. The amount of each compound was quantified by reference to a calibration curve, which was determined before each set of samples was run.

Starch Measurements

The starch content of each tissue was determined by extracting samples of 0.1 to 2 g fresh weight with ethanol then hydrolyzing the starch to Glc as described in Stitt et al. (1978).

ACKNOWLEDGMENTS

Dr. Kim Hammond-Kosack (Sainsbury Laboratory, Norwich, UK) donated tomato plants. Dr. Steve Rawsthorne (John Innes Centre) donated oilseed rape embryos. Dr. Glyn Harper and Dr. Rob Briddon (John Innes Centre) donated *Dioscorea bulbifera* and taro. Seeds of *Lolium temulentum* and *Avena fatua* were a gift from the Institute of Grassland and Environmental Research (Aberystwyth, Wales). Seeds of oat, barley, and wheat were a gift from the National Institute of Agricultural Botany (Cambridge, UK). Seeds of *Eleusine coracana* were a gift from Dr. Mike Ambrose (John Innes Centre). We thank Prof. Curt Hannah (University of Florida, Gainesville) for the gift of the BRITTLE2 antiserum, Prof. Roland von Bothmer (Swedish University of Agricultural Science, Svalov, Sweden), and Dr. Shin Taketa (University of Okayama, Japan) for their advice on *Hordeum* taxonomy and history, and Mr. Alexander Goodall (Botanic Gardens, University of Cambridge, UK) for cultivating many of the plants. D.M.B. thanks Prof. E. MacRobbie (University of Cambridge) for her support.

Received July 24, 2000; returned for revision September 5, 2000; accepted October 5, 2000.

LITERATURE CITED

- ap Rees T** (1974) Pathways of carbohydrate breakdown in plants. In DH Northcote, ed, MTP International Review of Science: Biochemistry, Series One, Vol 11. Butterworths, London, pp 89–129
- ap Rees T** (1988) Hexose phosphate metabolism by non-photosynthetic tissues of higher plants. In J Preiss, ed, The Biochemistry of Plants, Vol 14. Academic Press, New York, pp 1–33
- ap Rees T** (1995) Where do plants make ADPglucose? In HG Pontis, GL Salerno, EJ Echeverria, eds, Sucrose: Metabolism, Biochemistry, Physiology and Molecular Biology. American Society of Plant Physiologists, Rockville, MD, pp 143–155
- ap Rees T, Leja M, Macdonald FD, Green JH** (1984) Nucleotide sugars and starch synthesis in the spadix of *Arum maculatum* and suspension cultures of *Glycine max*. *Phytochemistry* **23**: 2463–2468
- Bhattacharyya MK, Smith AM, Ellis THN, Hedley C, Martin C** (1990) The wrinkled seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch branching enzyme. *Cell* **60**: 115–122
- Borchert S, Harborth J, Schünemann D, Hoferichter P, Heldt HW** (1993) Studies of the enzymic capacities and transport properties of pea root plastids. *Plant Physiol* **101**: 303–312
- Brangeon J, Reyss A, Prioul JL** (1997) In situ detection of ADPglucose pyrophosphorylase expression during maize endosperm development. *Plant Physiol Biochem* **35**: 847–858
- Chen BY, Wang Y, Janes HW** (1998) ADP-glucose pyrophosphorylase is localized to both the cytoplasm and plastids in developing pericarp of tomato fruit. *Plant Physiol* **116**: 101–106
- Denyer K, Dunlap F, Thorbjørnsen T, Keeling P, Smith AM** (1996) The major form of ADPglucose pyrophosphorylase in maize endosperm is extraplastidial. *Plant Physiol* **112**: 779–785
- Denyer K, Smith AM** (1988) The capacity of plastids from developing pea cotyledons to synthesize acetyl CoA. *Planta* **173**: 172–182
- Echeverria E, Boyer C** (1986) Localization of starch biosynthetic and degradative enzymes in maize leaves. *Am J Bot* **73**: 167–171
- Edwards A, Marshall J, Sidebottom C, Visser RGF, Smith AM, Martin C** (1995) Biochemical and molecular characterization of a novel starch synthase from potato tubers. *Plant J* **8**: 283–294
- Edwards J, ap Rees T** (1986) Metabolism of UDPglucose by developing embryos of round and wrinkled varieties of *Pisum sativum*. *Phytochemistry* **25**: 2033–2039
- Edwards J, Green JH, ap Rees T** (1988) Activity of branching enzyme as a cardinal feature of the *Ra* locus in *Pisum sativum*. *J Exp Bot* **38**: 1781–1788
- Geigenberger P, Merlo L, Reimholz R, Stitt M** (1994) When growing potato tubers are detached from their mother plant there is a rapid inhibition of starch synthesis, involving inhibition of ADP-glucose pyrophosphorylase. *Planta* **193**: 486–493
- Geigenberger P, Stitt M** (1993) Sucrose synthase catalyzes a readily reversible reaction in vivo in developing potato tubers and other plant tissues. *Planta* **189**: 329–339
- Giroux M, Hannah LC** (1994) ADP-glucose pyrophosphorylase in *shrunk-2* and *brittle-2* mutants of maize. *Mol Gen Genet* **243**: 400–408
- Gross P, ap Rees T** (1986) Alkaline inorganic pyrophosphatase and starch synthesis in amyloplasts. *Planta* **167**: 140–145
- Jenner CF, Denyer K, Hawker JS** (1994) Caution in the use of the generally accepted methanol precipitation technique for the assay of soluble starch synthase in crude extracts of plant tissues. *Aust J Plant Physiol* **21**: 17–22
- Kang F, Rawsthorne S** (1994) Starch and fatty acid synthesis in plastids from developing embryos of oilseed rape (*Brassica napus* L.). *Plant J* **6**: 795–805
- Kleczkowski LA** (1994) Glucose activation and metabolism through UDP-glucose pyrophosphorylase in plants. *Phytochemistry* **37**: 1507–1515
- Kleczkowski LA, Villand P, Lüthi E, Olsen OA, Preiss J** (1993) Insensitivity of barley endosperm ADP-glucose pyrophosphorylase to 3-phosphoglycerate and orthophosphate regulation. *Plant Physiol* **101**: 179–186
- Laemmli UK** (1970) Cleavage of structural proteins during assembly of bacteriophage T4. *Nature* **227**: 680–685

- Miller ME, Chourey PS** (1995) Intracellular immunolocalization of adenosine 5'-diphosphoglucose pyrophosphorylase in developing endosperm cells of maize (*Zea mays* L.). *Planta* **197**: 522–527
- Naeem M, Tetlow IJ, Emes MJ** (1997) Starch synthesis in amyloplasts purified from developing potato tubers. *Plant J* **11**: 1095–1103
- Okita TW, Greenberg E, Kuhn DN, Preiss J** (1979) Subcellular localization of the starch degradative and biosynthetic enzymes of spinach leaf. *Plant Physiol* **64**: 187–192
- Plaxton WC, Preiss J** (1987) Purification and properties of non-proteolytic degraded ADPglucose pyrophosphorylase from maize endosperm. *Plant Physiol* **83**: 105–112
- Robinson NL, Preiss J** (1987) Localization of carbohydrate metabolizing enzymes in guard cells of *Commelina communis*. *Plant Physiol* **85**: 360–364
- Shannon JC, Pien FM, Cao H, Liu KC** (1998) Brittle-1, an adenylate translocator, facilitates transfer of extraplasmidial synthesized ADPglucose into amyloplasts of maize endosperms. *Plant Physiol* **117**: 1235–1252
- Shannon JC, Pien FM, Liu KC** (1996) Nucleotides and nucleotide sugars in developing maize endosperms. *Plant Physiol* **110**: 835–843
- Smith AM** (1988) Major differences in isoforms of starch-branching enzyme between developing embryos of round- and wrinkled-seeded peas. *Planta* **175**: 270–279
- Smith AM, Bettey M, Bedford ID** (1989) Evidence that the *rb* locus alters the starch content of developing pea embryos through an effect on ADP glucose pyrophosphorylase. *Plant Physiol* **89**: 1279–1284
- Stitt M, Bulpin PV, ap Rees T** (1978) Pathway of starch breakdown in photosynthetic tissue of *Pisum sativum*. *Biochim Biophys Acta* **544**: 200–214
- Sweetlove LJ, Burrell MM, ap Rees T** (1996) Characterization of transgenic potato (*Solanum tuberosum*) tubers with increased ADPglucose pyrophosphorylase. *Biochem J* **320**: 487–492
- Tetlow IJ, Blissett KJ, Emes MJ** (1993) A rapid method for the isolation of purified amyloplasts from wheat endosperm. *Planta* **189**: 597–600
- Thorbjørnsen T, Villand P, Denyer K, Olsen OA, Smith AM** (1996) Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. *Plant J* **10**: 243–250
- Tyynelä J, Stitt M, Lønneborg A, Smeekens S, Schulman AH** (1995) Metabolism of starch synthesis in developing grains of the *shx* shrunken mutant of barley (*Hordeum vulgare*). *Physiol Plant* **93**: 77–84