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WHEAT STARCH BIOSYNTHESIS: ORGAN-SPECIFIC EXPRESSION OF GENES ENCODING ADP-GLUCOSE PYROPHOSPHORYLASE

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DECLARATION

The work contained in this thesis was the result of original research conducted by myself and has not been submitted in any previous application for a degree. All sources of information have been acknowledged by means of reference.

24/8/88

Mark Olive

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ABBREVIATIONS

ABTS	di-ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
AMV	avian myeloblastosis virus
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin (fraction V)
bp	base pairs
cDNA	complementary DNA
c.p.m.	counts per minute
CTAB	hexadecyltrimethylammonium bromide
C-terminus	carboxy terminus
CTP	cytidine 5'-triphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate
DEAE	di-ethyl-amino-ethyl
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	methyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
d.p.a.	days post anthesis
DTT	di-thiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate

EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbant assay
FP	free polysomes
FPLC	fast protein liquid chromatography
GTP	guanosine 5'-triphosphate
Ig	immunoglobulin
IPTG	isopropylthio-β-galactoside
kb	kilobasepairs
kD	kiloDaltons
MBP	membrane-bound polysomes
mRNA	messenger RNA
M.W.	molecular weight
NADP	nicotinamide-adenine dinucleotide phosphate
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced
N-terminus	amino terminus
PEG	polyethylene glycol
p.f.u.	plaque-forming units
3-PGA	3-phosphoglycerate
P_i	orthophosphate
PMSF	phenyl methane sulphonyl fluoride
Poly(A)	polyadenylated
PP_i	pyrophosphate
RNA	ribonucleic acid
RNase	ribonuclease
Rubisco	ribulose-1,5-bisphosphate carboxylase-oxygenase
SDS	sodium dodecylsulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
UDP	uridine 5'-diphosphate

UTP	uridine 5'-triphosphate
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

ABSTRACT

Wheat endosperm ADP-glucose pyrophosphorylase has been purified approximately 70-fold by two different procedures. The enzyme is composed of four subunits of identical molecular weight (51,000). Antibodies prepared against the denatured 51 kD subunit of wheat endosperm ADP-glucose pyrophosphorylase recognise a 48 kD polypeptide in soluble protein extracts of wheat leaves, suggesting that wheat leaf and wheat endosperm ADP-glucose pyrophosphorylases may be the products of different genes.

Two primary translation products of wheat endosperm ADP-glucose pyrophosphorylase mRNA have been identified by immunoprecipitation of the nascent polypeptides with anti-wheat endosperm ADP-glucose pyrophosphorylase serum. The ADP-glucose pyrophosphorylase primary translation products have apparent molecular weights of 61,000 and 53,000. The 61 kD polypeptide is found associated with membrane-bound polysomal RNA from wheat endosperm, while the smaller 53 kD polypeptide has been found associated almost exclusively with free polysomes.

A cDNA clone (WL:AGA.1) encoding wheat leaf ADP-glucose pyrophosphorylase has been isolated from a λgt11 expression library, by immunological screening with anti-spinach leaf ADP-glucose pyrophosphorylase serum. The WL:AGA.1 cDNA is 948 bp long and contains approximately 55% of the complete wheat leaf ADP-glucose pyrophosphorylase mRNA sequence, estimated from Northern blot experiments. A wheat endosperm cDNA library, consisting of $1.6x10^{8}$ bacteriophage particles, was subsequently constructed in the λgt11 expression vector. Six clones hybridising to the cDNA insert of clone WL:AGA.1 were isolated from the wheat endosperm cDNA library. The longest of these wheat endosperm ADP-glucose pyrophosphorylase cDNAs, clone WE:AGA.7, is nearly full-length (1798 bp), indicated by Northern blot analysis of wheat endosperm mRNA, coupled *in vitro* transcription and translation of the cDNA, and nucleotide sequence analysis.

Southern hybridisation analysis and restriction enzyme mapping indicated that the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase cDNAs and genes are members of two distinct sub-families, expressed in an organ-specific manner. Southern blot analysis of wheat genomic DNA has been performed using the WE:AGA.7 and WL:AGA.1 cDNA inserts as probes. This analysis indicated that each gene sub-family consists of approximately 2-5 genes. In addition, restriction enzyme mapping of wheat endosperm ADP-glucose pyrophosphorylase cDNAs revealed the presence of two classes of that sequence, indicating the existence of at least two wheat endosperm ADP-glucose pyrophosphorylase genes.

Subsequent nucleotide sequence comparison of wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase cDNAs indicates that there is approximately 55% identity between them. In contrast, the wheat endosperm cDNAs are very closely related. Members of each class of endosperm cDNA, represented by clones WE:AGA.3 and WE:AGA.7, are 96% identical.

The derived amino acid sequences of wheat leaf and endosperm ADP-glucose pyrophosphorylases were determined from the nucleotide sequences of the corresponding cDNAs. These sequences are approximately 24% identical to the amino acid sequences of *E.coli* ADP-glucose pyrophosphorylase and 40% identical to the rice endosperm ADP-glucose pyrophosphorylase. The structure-function relationships of substrate, activator, and inhibitor binding sites on the ADP-glucose pyrophosphorylase enzyme are discussed.

SECTION I - LITERATURE REVIEW

1. INTRODUCTION

Starch is present in every organ of higher plants, for example in the leaves, seeds, roots, shoots, stems and pollen grains. Starch is an important end-product of carbon fixation during photosynthesis in leaves and an important storage product in seeds and fruits. In economic terms, the starch produced by the edible portions of three grain crops, wheat, rice and maize, provide approximately two-thirds of the world's food calculated as calories (Jenner, 1982).

Starch accumulating in the endosperm of cereals is synthesised from the assimilates and stored carbohydrates made by the flag leaves. In wheat, the assimilates by the ear stay mostly in the grain and also contribute to total stored starch in the endosperm (Yoshida, 1972). In addition to this relationship, several organ-specific processes can be discerned with respect to the patterns of starch accumulation in plants. In leaves, the starch formed during the day is degraded by respiration during periods of low light intensity or darkness (Sachs, 1887, cited by Preiss and Levi, 1980). Thus, the processes of starch biosynthesis and breakdown in plant leaves are closely associated, in a diurnal cycle. In non-photosynthetic plant organs there are no detectable diurnal variations in starch metabolism. In the endosperm of seeds, starch accumulates over a period of several weeks, terminating at maturity (Jenner, 1982). The metabolic and catabolic processes are temporally disjoint in storage organs, with starch degradation accompanying distinct physiological changes such as germination or sprouting.

Starch is synthesised in the plastid compartment i.e. the chloroplast in photosynthetic cells or the amyloplast in non-photosynthetic cells. The biochemical pathway of starch biosynthesis in leaves has been well-characterised, and is presented in Section I.2.A. In contrast, the absence of a method for the isolation of intact amyloplasts from endosperm cells until recently, has prevented the elucidation of the pathway of starch biosynthesis in that plant organ. Recent research on starch biosynthesis in wheat endosperm indicates that, just as the patterns of starch accumulation differ in the leaves and storage organs, there is a similar organ-specificity in the biochemical pathways of starch biosynthesis and the mechanisms regulating starch biosynthesis in cereals. This chapter reviews the current information on the biochemical pathways and regulation of starch biosynthesis in the leaves and endosperm of plants. In view of the economic importance of cereal starch, particular emphasis has been placed on starch biosynthesis in wheat endosperm.

2. STARCH BIOSYNTHESIS IN LEAVES

A. The biochemical pathway of starch biosynthesis in leaves

Chloroplasts contain all the enzymes required for the biosynthesis of leaf starch. In C-3 plants, the direct fixation of CO_2 produces 3-phosphoglycerate which serves as a substrate for starch biosynthesis in the chloroplast, and sucrose biosynthesis in the cytosol (see Figure I.1). In C-4 plants the substrate for starch biosynthesis is also 3-phosphoglycerate; however starch

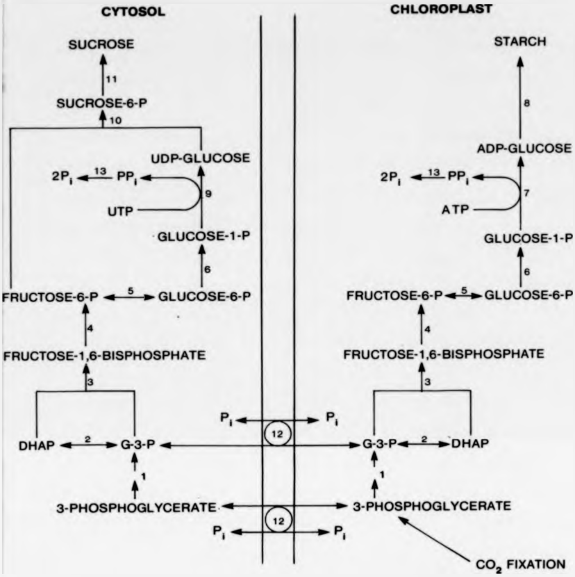

CYTOSOL
CHLOROPLAST
SUCROSE
11
SUCROSE-6-P
10
UDP-GLUCOSE
$2P_i$
13
PP_i
9
UTP
GLUCOSE-1-P
6
FRUCTOSE-6-P
5
GLUCOSE-6-P
4
FRUCTOSE-1,6-BISPHOSPHATE
3
DHAP
2
G-3-P
1
3-PHOSPHOGLYCERATE
P_i
12
P_i
12
P_i
P_i
STARCH
8
ADP-GLUCOSE
$2P_i$
13
PP_i
7
ATP
GLUCOSE-1-P
6
FRUCTOSE-6-P
5
GLUCOSE-6-P
4
FRUCTOSE-1,6-BISPHOSPHATE
3
G-3-P
2
DHAP
1
3-PHOSPHOGLYCERATE
CO_2 FIXATION

Figure I.1 : The reactions involved in the biosynthetic pathways of starch and sucrose in leaves (adapted from Preiss, 1984). Abbreviations used are : G-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; P_i, orthophosphate; PP_i, inorganic pyrophosphate. The reactions are catalysed by the following enzymes :

1) phosphoglycerate kinase/ glyceraldehyde-3-phosphate dehydrogenase
2) triose-phosphate isomerase
3) aldolase
4) fructose-1,6-bisphosphatase
5) hexose phosphate isomerase
6) phosphoglucomutase
7) ADP-glucose pyrophosphorylase
8) starch synthase
9) UDP-glucose pyrophosphorylase
10) sucrose phosphate synthase
11) sucrose phosphatase
12) orthophosphate/ triose-phosphate translocator
13) inorganic pyrophosphatase

biosynthesis occurs principally in bundle sheath chloroplasts where 3-phosphoglycerate is produced via the decarboxylation of malate or aspartate (Kelly *et al*, 1976). The 3-phosphoglycerate is converted to α-D-glucose-6-phosphate in the chloroplast by enzymes of the pathway of gluconeogenesis i.e. phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, aldolase, fructose-1,6-bisphosphatase and hexose phosphate isomerase. The subsequent conversion of α-D-glucose-6-phosphate to α-D-glucose-1-phosphate is catalysed by the enzyme, phosphoglucomutase (see Figure I.1).

Enzymes catalysing the reactions invovled in the conversion of 3-phosphoglycerate to α-D-glucose-1-phosphate are present in both the cytosol and the chloroplast (Figure I.1). Since 3-phosphoglycerate serves as a substrate for starch biosynthesis in the chloroplast and sucrose biosynthesis in the cytosol, and α-D-glucose-1-phosphate is an intermediate in both biochemical pathways, it is likely that the enzyme-catalysed reactions from 3-phosphoglycerate to α-D-glucose-1-phosphate are the same in the biosynthesis of starch and the biosynthesis of sucrose. However, the enzymes themselves are not identical in the plastid and cytosol. Different isoenzymes of fructose-1,6-bisphosphatase for example, are present in the plastid and the cytosol; cytosolic fructose-1,6-bisphosphatase is allosterically inhibited by fructose-2,6-bisphosphate, while the plastid enzyme is inhibited competitively by fructose-2,6-bisphosphate (Herzog *et al*, 1984; Gottschalk *et al*, 1982; Cadet, 1987). In addition the plastid and cytosolic isoenzymes of fructose-1,6-bisphosphatase have different Km values for substrates, pH optima and Mg^{2+} requirements (Stitt *et al*, 1982).

As shown in Figure I.1, the biochemical pathway from α-D-glucose-1-phosphate to starch involves two plastid-specific enzyme-catalysed reactions. These two steps in the biosynthesis of starch are common to chloroplasts and amyloplasts. The biosynthesis of α-1,4 glucosidic linkages in starch is catalysed by the starch synthase enzyme (Figure I.1, reaction 8; Leloir *et al*, 1961). Although the starch synthase reaction was originally described by Leloir *et al* (1961) using UDP-glucose as the glucosyl donor, it was subsequently shown that ADP-glucose is the preferred substrate of the enzyme, in terms of V_{max} and Km values (Recondo and Leloir, 1961). Since then, several lines of evidence indicate that ADP-glucose is the glucosyl donor in starch biosynthesis in both chloroplasts and amyloplasts. Firstly, the soluble and granule-bound starch synthase enzymes from spinach chloroplasts are only active with ADP-glucose or deoxy-ADP-glucose as a substrate (Murata and Akazawa, 1964; Ghosh and Preiss, 1964). Secondly, the transfer of glucose to starch catalysed by cereal endosperm granule-bound starch synthases occurs 3-10 times more rapidly from ADP-glucose than from UDP-glucose (Murata *et al*, 1964). The granule-bound starch synthase enzymes from most plant storage organs examined to date have Km values for ADP-glucose which are 15-30 fold less than the Km values for UDP-glucose (Preiss and Levi, 1980). In addition, the soluble starch synthase enzymes from storage organs are specific for ADP-glucose, with Km values in the range of 0.1-0.3 mM for ADP-glucose (Preiss and Walsh, 1981).

ADP-glucose is synthesised from α-D-glucose-1-phosphate by the ADP-glucose pyrophosphorylase (E.C.2.7.7.27) reaction (Figure I.1, reaction 7; Espada, 1962). The involvement of ADP-glucose pyrophosphorylase in the starch biosynthetic pathways of both photosynthetic organs and non-photosynthetic organs is supported by two lines of evidence. Lin *et al* (1988) have identified a mutant of *Arabidopsis thaliana* (L.) Heynh which contains less than 2% (w/w) of the starch of wild-type plants when grown under a normal 12 hr photoperiod. In either continuous light or elevated CO_2 concentrations, the mutant plant lacks starch in the leaves, roots, petiole, flower stalk and flower. The leaves of the mutant contain normal levels of starch synthase and UDP-glucose pyrophosphorylase activities, but less than 2% of the ADP-glucose pyrophosphorylase activity of normal plants. This finding indicates that ADP-glucose pyrophosphorylase is responsible for most, if not all, of the ADP-glucose synthesis in chloroplasts. Thus, the sucrose synthase enzyme is probably not involved in the production of ADP-glucose from sucrose in the cytosol in the pathway of starch biosynthesis in plants. In addition, since the *Arabidopsis thaliana* mutant contains elevated levels of starch phosphorylase (about twice the level found in normal plants, Lin *et al*, 1988) then a more direct enzymatic route from α-D-glucose-1- phosphate to starch, catalysed by that enzyme, is also excluded.

Mutant lines of maize which are deficient in the endosperm ADP-glucose pyrophosphorylase activity have also been reported (Tsai and Nelson, 1966). In mutants homozygous for either the *shrunken*-2 (*sh*-2) mutant alleles on chromosome 3, or the *brittle*-2 (*bt*-2) mutant alleles on chromosome 4, there is only about 5% of the ADP-glucose pyrophosphorylase activity present in normal endosperms (Dickinson and Preiss, 1969). Furthermore, Hannah and Nelson (1975) have shown that ADP-glucose pyrophosphorylase activity in maize endosperm increases with dosage of functional alleles at the *sh*-2 and *bt*-2 loci, indicating that these loci are structural genes for this enzyme in maize. The reduction in ADP-glucose pyrophosphorylase activity in the endosperm of maize plants carrying *sh*-2 or *bt*-2 mutant alleles is correlated with a reduction in starch content. Tsai and Nelson (1966) reported that these mutants have only 25% (w/w) of that found in normal maize endosperm.

B. Regulation of starch biosynthesis in leaves at the level of ADP-glucose pyrophosphorylase

1. Allosteric regulation of ADP-glucose pyrophosphorylase

The chloroplast enzyme, ADP-glucose pyrophosphorylase, is regulated post-translationally by the allosteric effectors 3-phosphoglycerate and orthophosphate. All leaf ADP-glucose pyrophosphorylase enzymes studied (Copeland and Preiss, 1981; MacDonald and Strobel, 1970; Sanwal *et al*, 1968; Spilatro and Preiss, 1987), and the enzymes from *Chlorella vulgaris* (Nakamura and Immamura, 1985), *Synechococcus* 6301 (Levi and Preiss, 1976), potato tubers (Sowokinos, 1981; Sowokinos and Preiss, 1982) and maize endosperm (Plaxton and Preiss, 1987) are activated *in vitro* by 3-phosphoglycerate (see Table I.1). The extent of activation obtained varies between species, but generally enzyme activity is stimulated up to 30-fold above the levels obtained in the absence of 3-phosphoglycerate (Table I.1). In contrast, wheat endosperm ADP-glucose pyrophosphorylase is not allosterically activated by

Table I.1: Kinetic parameters for activation and inhibition of ADP-glucose pyrophosphorylase enzymes from various sources

Enzyme source	$^1A_{0.5}$ 3-PGA (μM)	^{2}Activation (-fold)	$^3I_{0.5}$ P_i (μM)	$^4I_{0.5}$ P_i (μM)	^{5}3-PGA conc. (mM)	Reference
1. PLANT LEAVES :						
Barley	7.0	15.0	20	2,300	1.4	Sanwal *et al* (1968)
Maize -mesophyll	950.0	4.2	n.d.	260	2.0	Spilatro and Preiss (1987)
- bundle sheath	490.0	13.8	n.d.	260	2.0	Spilatro and Preiss (1987)
Rice	180.0	15.0	60	270	1.0	Sanwal *et al* (1968)
Sorghum	370.0	8.0	190	410	2.2	Sanwal *et al* (1968)
Spinach	51.0	20.0	45	970	1.0	Copeland and Preiss (1981)
Sugarbeet	190.0	10.0	50	430	0.87	Sanwal *et al* (1968)
Tobacco	45.0	10.0	30	1,010	1.0	Sanwal *et al* (1968)
Wheat	100.0	14.5	56	n.d.	-	MacDonald and Strobel (1970)
2. BLUE-GREEN BACTERIA :						
Synechococcus	112.0	21.0	72	1,000	1.0	Levi and Preiss (1976)
3. GREEN ALGAE :						
Chlorella vulgaris	410.0	10.0	1,000	1,000	2.0	Nakamura and Immamura (1985)
4. PLANT STORAGE ORGANS						
Potato tuber	400.0	32.7	n.d.	180	0.5	Sowokinos and Preiss (1982)
Maize endosperm	120.0	20.6	N/A	440	1.0	Plaxton and Preiss (1987)
Rice endosperm	5,000.0	1.2	700	2,300	1.0	Lee and Wang (1985)
Wheat endosperm	N/A	0	700	1,500	1.0	Fentem and Catt (pers. comm.)

1. $A_{0.5}$ refers to the concentration of 3-phosphoglycerate required to achieve 50% maximal stimulation of enzyme activity *in vitro*.
2. The extent of activation obtained using 1 mM 3-phosphoglycerate in the assay conditions described by the authors.
3. $I_{0.5}$ refers to the concentration of orthophosphate required to achieve 50% inhibition of the enzyme activity in the absence of 3-phosphoglycerate.
4. The $I_{0.5}$ value for orthophosphate in the presence of added 3-phosphoglycerate.
5. The concentration of 3-phosphoglycerate used to determine the $I_{0.5}$ value shown in column 4.

N/A indicates that the metabolite has no effect on *in vitro* enzyme activity.

n.d. indicates that the parameter has not been calculated by the authors listed.

3-phosphoglycerate (see Section I.3.B.2).

The glycolytic intermediates phosphoenolpyruvate, fructose-6-phosphate, and fructose-1,6-bisphosphate activate the ADP-glucose pyrophosphorylase enzymes from leaves to lesser extents than does 3-phosphoglycerate, producing a maximum of 8-fold stimulation of all of the enzymes listed in Table I.1. These metabolites all achieve a stimulation of enzyme activity by lowering the Km values of substrates and increasing the V_{max} of the enzyme-catalysed reaction (Preiss 1982). Other glycolytic intermediates, such as di-hydroxy-acetone phosphate, glyceraldehyde-3-phosphate and pyruvate have not been convincingly demonstrated to stimulate ADP-glucose pyrophosphorylase activity *in vitro*. In addition, the ADP-glucose pyrophosphorylase enzymes from leaves are inhibited *in vitro* by orthophosphate, with half-maximal inhibition of enzyme activity generally achieved in orthophosphate concentrations of less than 100 μM (Table I.1)

The presence of both 3-phosphoglycerate and orthophosphate in assays of ADP-glucose pyrophosphorylase activity yields sigmoidal saturation curves (Sanwal *et al*, 1968; Ghosh and Preiss, 1966). For the ADP-glucose pyrophosphorylase enzymes listed in Table I.1, with the exception of the enzyme from *Chlorella vulgaris* (Nakamura and Immamura, 1985), the presence of 3-phosphoglycerate can be demonstrated to modulate the sensitivity of the enzyme to inhibition by orthophosphate, evidenced by an increase in the $I_{0.5}$ values in the presence of the activator (see Table I.1).

2. The *in vivo* significance of allosteric regulation of chloroplastic ADP-glucose pyrophosphorylase

The first suggestions that ADP-glucose pyrophosphorylase might be involved in regulating leaf starch biosynthesis came from the demonstration that the rate of starch biosynthesis in chloroplasts is inversely related to the concentration of orthophosphate, a potent inhibitor of ADP-glucose pyrophosphorylase. Steup *et al* (1976) demonstrated that isolated, intact spinach chloroplasts incubated in the light, in media containing a range of orthophosphate concentrations, produce the highest rates of $^{14}CO_2$-incorporation into starch at levels below 0.25 mM orthophosphate. These authors also noted that inhibition of chloroplast starch biosynthesis occurred at concentrations greater than 0.5 mM orthophosphate. Increases in orthophosphate concentration of this order of magnitude could conceivably inhibit ADP-glucose pyrophosphorylase activity *in situ*, since the spinach leaf ADP-glucose pyrophosphorylase enzyme has an $I_{0.5}$ value for orthophosphate of 970 μM, in the presence of 1 mM 3-phosphoglycerate (Copeland and Preiss, 1981). Thus, the observed reductions in rates of starch biosynthesis at high concentrations of orthophosphate might have been explicable in terms of allosteric inhibition of ADP-glucose pyrophosphorylase.

In support of this hypothesis, MacDonald and Strobel (1970) have demonstrated that in *Puccinia striiformis*-infected wheat leaves, there is an inverse correlation between the concentrations of accumulated starch and orthophosphate, throughout the cycle of infection (MacDonald and Strobel, 1970). The starch content of diseased leaves was shown by MacDonald and Strobel (1970) to decline during the first 9 days following infection with *P.striiformis* (i.e. the flecking stage), and to increase 7- to 8-fold over the next 4 days (i.e. the sporulation stage); in contrast, orthophosphate concentrations increased during the flecking stage and decreased sharply during sporulation. It is thus possible that the fluctuations in starch content of wheat leaves throughout the period of infection with this parasite are caused by the fluctuating concentrations of orthophosphate, via allosteric modulation of ADP-glucose pyrophosphorylase activity. However, one must also consider that, because this system involves the use of diseased plants other factors might have been responsible for the variations in starch content of wheat leaves.

In order to understand the *in vivo* regulation of leaf starch biosynthesis, it is essential to understand the selective permeability of the chloroplast inner membrane, since leaf starch biosynthesis occurs within the chloroplast. Heldt and Rapley (1970) demonstrated that the inner membrane of chloroplasts is selectively permeable to triose-phosphates, dicarboxylates and orthophosphate, which are rapidly and specifically transported from the chloroplast to the cytosol and *vice versa*. In contrast, hexose-phosphates are not transported across the chloroplast membrane, suggesting that assimilated carbon must be transported from the plastid as triose-phosphate. This active transport mechanism involves the counter-balancing inward movement of orthophosphate from the cytosol via the phosphate/triose-phosphate translocator (Heldt and Rapley, 1970).

The active transport of metabolites via the orthophosphate/ triose-phosphate translocator is involved in altering the ratio of orthophosphate to 3-phosphoglycerate (i.e. $[P_i]/[3\text{-PGA}]$) within the plastid during normal light-dark transitions. Kaiser and Bassham (1979a) have demonstrated that the concentration of 3-phosphoglycerate increases in isolated spinach chloroplasts, from 1.6 mM in the dark ($[P_i]/[3\text{-PGA}] = 2.5$) to 4 mM in the light ($[P_i]/[3\text{-PGA}] = 1.0$). In those experiments, the chloroplasts maintained the internal orthophosphate concentration at 4 mM; however the concentration of orthophosphate in the medium was shown to decrease during the light cycle in molar equivalents corresponding to the total triose-phosphate exported. This result suggested that the orthophosphate imported from the medium was being rapidly absorbed in reactions such as ATP formation. The net decrease in extrachloroplastic orthophosphate concentration was shown by Kaiser and Bassham (1979a) to stop after 30 sec incubation in the dark. Kaiser and Bassham (1979b) subsequently assayed ADP-glucose pyrophosphorylase activity under the approximate conditions found within intact chloroplasts in the light and in the dark. A 6- to 7-fold reduction in enzyme activity was observed when the metabolite concentrations were altered from 4 mM 3-phosphoglycerate and 4 mM orthophosphate (light cycle approximation) to 1.4 mM 3-phosphoglycerate and 4 mM orthophosphate (dark cycle approximation). Thus, the interaction between activator and inhibitor in altering ADP-glucose pyrophosphorylase activity is of great importance *in vivo*, since changes in the chloroplastic concentrations of 3-phosphoglycerate and orthophosphate will modulate the enzyme's activity within the plastid, thereby constituting a sensitive system for the direct regulation of leaf starch biosynthesis.

During normal photosynthesis, the 3-phosphoglycerate formed from CO_2-fixation accumulates in the chloroplast. The concentration gradient of 3-phosphoglycerate from the inside of the chloroplast to the cytosol leads to the export of some 3-phosphoglycerate into the cytosol in exchange for orthophosphate. The imported orthophosphate is subsequently utilised in ATP formation via photophosphorylation, so that the overall chloroplastic ratio of $[P_i]/[3\text{-PGA}]$ remains low during the light cycle. Thus the ADP-glucose pyrophosphorylase enzyme is allosterically activated in the light, allowing starch biosynthesis to continue. During the dark cycle, there is decreased CO_2-fixation and decreased photophosphorylation, coupled with increased chloroplastic orthophosphate concentration as a result of the hydrolysis of ATP. This produces a high chloroplastic ratio of $[P_i]/[3\text{-PGA}]$, thereby inhibiting ADP-glucose pyrophosphorylase activity and starch formation.

In addition to the modulation of ADP-glucose pyrophosphorylase activity by allosteric effectors, leaf starch biosynthesis is probably regulated by the adenylate energy charge of the chloroplast, also at the level of ADP-glucose pyrophosphorylase. Kaiser and Bassham (1979b) have also studied the effect of changes in ATP concentration on the formation of ADP-glucose in spinach chloroplast extracts. At concentrations of orthophosphate and 3-phosphoglycerate as they exist in chloroplasts in the light, an increase in the ATP concentration from 0.2 mM to 1.0 mM stimulates ADP-glucose pyrophosphorylase activity 4- to 5-fold. This change in ATP concentration is the same as that observed in chloroplasts during the dark to light transition (Kaiser and Urbach, 1973). Since the activation obtained with ATP under these conditions is greater than that obtained with the ratio of $[P_i]/[3\text{-PGA}]$ present in illuminated chloroplasts (Kaiser and Bassham, 1979a), the concentrations of both ATP and allosteric effectors are probably important in regulating the *in vivo* rate of starch biosynthesis. The Km of spinach leaf ADP-glucose pyrophosphorylase for ATP, in the presence of 1 mM 3-phosphoglycerate is 62 μM at pH 8.0 (Copeland and Preiss, 1981). However, orthophosphate is a mixed inhibitor of ADP-glucose pyrophosphorylase with respect to ATP, in the presence of 3-phosphoglycerate. The apparent Km of spinach leaf ADP-glucose pyrophosphorylase for ATP is 0.3 mM, in the presence of 2 mM 3-phosphoglycerate and 1.5 mM orthophosphate (Ghosh and Preiss, 1966). Thus, when the ratio of $[P_i]/[3\text{-PGA}]$ is low, the low chloroplastic concentration of ATP, which occurs during the dark, is probably insufficient to activate ADP-glucose pyrophosphorylase. The rise in chloroplastic ATP concentration occurs within 5-15 sec of illumination (Kaiser and Urbach, 1973), compared with 2-3 min for the concentration of 3-phosphoglycerate to increase to 4 mM (Kaiser and Bassham, 1979a). Since the rise in ATP concentration within the chloroplast precedes the rise in 3-phosphoglycerate upon illumination, then activation of ADP-glucose pyrophosphorylase during the first few minutes of illumination may depend solely upon the chloroplastic concentration of ATP . This effect may constitute a mechanism for the rapid activation of ADP-glucose pyrophosphorylase *in vivo*.

3. Regulation of leaf starch biosynthesis by the rate of sucrose formation

An interesting consequence of the compartmentalisation of starch biosynthesis in the chloroplast is that the rate of starch formation in leaves is indirectly regulated by the rate of sucrose formation. On one level, starch biosynthesis and sucrose biosynthesis are regulated by the same metabolites; orthophosphate will inhibit sucrose phosphate synthase as well as ADP-glucose pyrophosphorylase. In addition, the formation of fructose-2,6-bisphosphate, an inhibitor of cytosolic fructose-1,6-bisphosphatase activity, is inhibited by 3-phosphoglycerate and activated by orthophosphate (Preiss, 1984).

Because starch biosynthesis and sucrose biosynthesis are linked to photosynthesis and utilise the same carbon source (triose-phosphate), increased sucrose formation is generally associated with decreased starch formation and *vice versa*. Sucrose formation is regulated at the level of sucrose phosphate synthase, which catalyses the essentially irreversible formation of sucrose phosphate from UDP-glucose and fructose-6-phosphate (Figure I.1; Huber, 1983; Huber and Israel, 1982; Salerno and Pontis, 1978). A high concentration of sucrose phosphate synthase in leaf extracts has been correlated with greater sucrose formation and reduced starch accumulation in four varieties of soybean (Huber and Israel, 1982), and four separate species (wheat, soybean, tobacco, and red beet), as well as in nine peanut cultivars (Huber, 1983). Huber and Israel (1982) have subsequently postulated that sucrose phosphate synthase is rate-limiting with respect to sucrose formation and that the rate of conversion of phosphate esters to sucrose will control the cytoplasmic pool size of orthophosphate and triose-phosphates. In plants with increased sucrose phosphate synthase, a greater conversion of cytosolic triose-phosphates to sucrose would lead to increased cytosolic levels of orthophosphate, produced by the sucrose phosphatase, UDP-glucose pyrophosphorylase and fructose-1,6- bisphosphatase reactions. The increase in orthophosphate concentration within the cytosol would subsequently produce an increased flux of triose-phosphate out of the chloroplast and into the cytosol in exchange for orthophosphate, thereby limiting the amount of carbon available for starch biosynthesis.

However, it is simplistic to assume that complex biochemical pathways are regulated by a single 'rate-limiting enzyme', under a variety of environmental conditions, substrate pool sizes, etc. It is more likely that the flux through any biochemical pathway is regulated by more than one enzyme, thereby enabling the flux to adapt to different external parameters. This concept was originally postulated by Kacser and Burns (1973), who developed a quantitative approach to the analysis of the control of flux of metabolites in biochemical pathways, in an attempt to determine the relative importance of certain enzymes as flux regulators. Recently, M. Stitt and co-workers at the University of Bayreuth have analysed the factors controlling carbon flux from CO_2 to sucrose in *Clarkia* plants, using the control strength analysis of Kacser and Burns (1973). The results of that analysis suggest that the control of sucrose biosynthesis is shared between the enzymes sucrose phosphate synthase and cytosolic fructose-1,6-bisphosphatase; at high light intensities the concentration of sucrose phosphate synthase regulates sucrose biosynthesis, while at low light intensities control shifts to fructose-1,6-bisphosphatase (cited in Bendall and Gray, 1988). Thus, the concentration of cytosolic fructose-1,6-bisphosphatase may also be able to regulate the partitioning of carbon between sucrose and starch, and thereby indirectly control starch biosynthesis in chloroplasts. In addition, Stitt and co-workers have demonstrated that the concentrations of cytosolic and chloroplastic hexose phosphate isomerases regulate carbon partitioning in *Clarkia* plants. In *Clarkia* mutants containing reduced chloroplastic hexose phosphate isomerase activity, there is reduced flux of carbon to starch, while in mutants containing reduced cytosolic hexose phosphate isomerase activity, there is reduced flux of carbon to sucrose (cited in Bendall and Gray, 1988).

3. STARCH BIOSYNTHESIS IN THE ENDOSPERM OF CEREALS

A. The biochemical pathway of starch biosynthesis in wheat endosperm

The starch present in endosperm is synthesised from sucrose which has been translocated from the photosynthetic cells of the flag leaves and the ear (Jenner, 1982). Since it is not my intention to deal with the pathway of sucrose biosynthesis in detail, the reader is referred to reviews on the subject by Avigad (1982), Preiss (1984), and Whittingham *et al* (1979). The pathway of sucrose biosynthesis, is presented in Figure I.1. The enzymes involved in catalysing the conversion of 3-phosphoglycerate to hexose-monophosphate in the biosynthesis of sucrose are located in the cytosol as well as the chloroplast. We might expect therefore, that the enzyme reactions of the sucrose biosynthetic pathway from 3-phosphoglycerate to hexose-monophosphate are identical to the steps in the formation of hexose-monophosphate in chloroplastic starch biosynthesis. The subsequent conversion of hexose-monophosphate to sucrose involves the action of UDP-glucose pyrophosphorylase, sucrose phosphate synthase and sucrose phosphatase, which are located in the cytosol (Figure I.1). The orthophosphate required to sustain the supply of triose-phosphate to the cytosol for sucrose biosynthesis is obtained from the fructose-1,6-bisphosphatase and sucrose phosphatase reactions, and also from the cleavage of pyrophosphate produced during the formation of UDP-glucose (Figure I.1).

The initial steps in the utilisation of sucrose for starch biosynthesis occur in the cytosol of endosperm cells and have been well-characterised. The involvement of the sucrose synthase enzyme in the direct conversion of sucrose to the sugar nucleotide, UDP-glucose, was indicated by studies on the *shrunken*-1 (*sh*-1) mutant of maize. The *shrunken* locus on chromosome 9 codes for the sucrose synthase enzyme and in maize lines which are homozygous for the *sh*-1 mutant allele, the endosperm contains less than 10% of the sucrose synthase activity present in normal endosperms (Chourey and Nelson, 1976). This enzyme deficiency reduces endosperm starch content to 60% (w/w) of the level found in normal maize endosperm (Chourey and Nelson, 1976). In addition, it is unlikely that there is an alternative pathway for the utilisation of sucrose via invertase, which hydrolyses sucrose to glucose plus fructose, as there is no invertase activity in the endosperm of wheat (Chevalier and Lingle, 1983). If the first step in the conversion of sucrose into starch involves the formation of UDP-glucose, then the second step probably involves the formation of α-D-glucose-1-phosphate, catalysed by UDP-glucose pyrophosphorylase. Recently, G. Entwhistle and T. ap Rees (Botany School, University of Cambridge, Cambridge, U.K.; personal communications) have shown that wheat endosperm UDP-glucose pyrophosphorylase activity is confined to the cytosol. Consequently, the initial metabolism of sucrose to α-D-glucose-1-phosphate occurs in the cytosol.

The remainder of the pathway was thought until recently to involve the conversion of α-D-glucose-1-phosphate to fructose-6-phosphate in the cytosol, via phosphoglucomutase and hexokinase-catalysed reactions. It was postulated that triose-phosphate would be subsequently formed in the cytosol of endosperm cells via glycolysis, and transported into the amyloplast via the orthophosphate/triose-phosphate translocator. In this scheme, triose-phosphate would follow the same route to starch as it does in the chloroplast of photosynthetic cells. However, recent studies on starch biosynthesis in wheat endosperm provide three lines of evidence to refute this hypothesis.

Firstly, Keeling *et al* (1988) have studied the redistribution of ^{13}C label between carbon-1 (C-1) and carbon-6 (C-6) of asymmetrically-labelled glucose incorporated into starch. In these experiments, only 15% of the label incorporated into starch was redistributed. Similar results were obtained when either [1-^{13}C]glucose or [1-^{13}C]fructose were supplied to endosperms. The small amount of label redistribution observed is believed to be the result of a futile cycle between hexose-monophosphate and triose-phosphate in the cytosol (Figure I.2), which serves to produce inorganic pyrophosphate for use by the UDP-glucose pyrophosphorylase enzyme. In wheat endosperm labelled with [1-^{13}C]glucose, Keeling *et al* (1988) observed a similar redistribution pattern between C-1 and C-6 in the glucosyl and fructosyl moieties of sucrose to the pattern found previously in starch. If the pathway from α-D-glucose-1-phosphate to starch

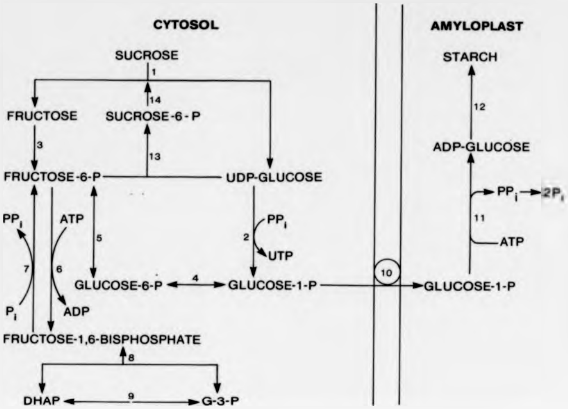

CYTOSOL
AMYLOPLAST
SUCROSE
1
14
FRUCTOSE
SUCROSE-6-P
3
13
FRUCTOSE-6-P
UDP-GLUCOSE
PP$_i$
ATP
5
2
PP$_i$
UTP
7
6
GLUCOSE-6-P
4
GLUCOSE-1-P
10
GLUCOSE-1-P
P$_i$
ADP
FRUCTOSE-1,6-BISPHOSPHATE
8
DHAP
9
G-3-P
STARCH
12
ADP-GLUCOSE
PP$_i$
2P$_i$
11
ATP

Figure I.2 : The proposed metabolic pathway of starch biosynthesis in wheat endosperm (from Keeling *et al*, 1988). The abbreviations used are the same as for figure I.1. The reactions are catalysed by the following enzymes :

1) sucrose synthase
2) UDP-glucose pyrophosphorylase
3) hexokinase
4) phosphoglucomutase
5) hexose-phosphate isomerase
6) ATP-dependent phosphofructokinase
7) PP$_i$-dependent phosphofructokinase
8) aldolase
9) triose-phosphate isomerase
10) hexose-phosphate translocator ?
11) ADP-glucose pyrophosphorylase
12) starch synthase
13) sucrose phosphate synthase
14) sucrose phosphatase

involved a triose-phosphate intermediate, the extent of label redistribution between C-1 and C-6 found in starch would significantly exceed the redistribution of label in the hexosyl moieties of sucrose. This observation therefore seriously weakens the case for direct involvement of a triose-phosphate intermediate in the pathway of starch biosynthesis in wheat endosperm.

Secondly, the isolation of intact amyloplasts from wheat endosperm has facilitated studies on the permeability of the amyloplast membrane to various metabolites. A method for the isolation of intact amyloplasts from wheat endosperm has recently been published (Entwhistle *et al*, 1988). Tyson and ap Rees (1988) have subsequently demonstrated that amyloplasts prepared in this way are functional in their ability to synthesise starch from externally-supplied, labelled intermediates. Supply of external [U-^{14}C]glucose-1-phosphate to intact amyloplasts was shown by Tyson and ap Rees (1988) to result in the incorporation of 95% of the total label into starch. This incorporation was totally dependent on the intactness of amyloplasts. Thus, α-D-glucose-1-phosphate must be imported into the wheat endosperm amyloplast. In contrast, these authors found no incorporation of label into starch when amyloplasts were supplied with ADP-[U-^{14}C]glucose, [U-^{14}C]glucose, [U-^{14}C]glucose-6-phosphate, [U-^{14}C]fructose-6-phosphate, [U-^{14}C] fructose-1,6-bisphosphate, [U-^{14}C]dihydroxyacetone phosphate, or [U-^{14}C]glycerol-3 phosphate. These observations suggest that carbon is not transported into the amyloplast as a triose-phosphate. It is not known at present whether α-D-glucose-1-phosphate is the sole carbon source imported into the wheat endosperm amyloplast for incorporation into starch.

The exact nature of the transport of α-D-glucose-1-phosphate into the amyloplast has not been elucidated. Further studies are required to determine whether an active transport mechanism is involved, and what factors are involved in the transport of metabolites across the amyloplast membrane. For example, if a hexose-phosphate translocator were involved in transport of a-D-glucose-1-phosphate into the amyloplast, then does this transport involve the counter movement of ions into the cytosol, by a similar mechanism as for the translocation of 3-phosphoglycerate and orthophosphate across the chloroplast membrane?

Finally, studies on the enzymic capacities of wheat endosperm amyloplasts indicate that these organelles do not contain fructose-1,6-bisphosphatase activity, or pyrophosphate-dependent phosphofructokinase activity (G. Entwhistle and T. ap Rees, personal communications). G. Entwhistle and T. ap Rees (personal communications) have also demonstrated that antibody to plastidic fructose-1,6-bisphosphatase does not react with wheat endosperm soluble protein, in Western blotting experiments. As a consequence of these findings, there is no evidence for a route within the amyloplast for the re-synthesis of hexose-monophosphate from triose-phosphates. This conclusion contrasts with studies on the enzymic capacities of amyloplasts isolated from soybean suspension cells. MacDonald and ap Rees (1983) demonstrated that soybean amyloplasts contain all of the enzymes needed to convert triose-phosphate to starch. However, the specific import of metabolites into soybean amyloplasts has not been demonstrated and therefore any one of the glycolytic intermediates from α-D-glucose-1-phosphate to triose-phosphate might be imported as a substrate for starch biosynthesis in soybean amyloplasts.

Thus, in wheat endosperm cells at least, α-D-glucose-1-phosphate produced in the cytosol crosses the amyloplast membrane and is converted to starch by the plastid-localised enzymes, ADP-glucose pyrophosphorylase and starch synthase (Figure I.2). It remains to be seen whether this pathway is ubiquitous to the non-photosynthetic storage organs of other plants.

B. Regulation of starch biosynthesis in the endosperm

1. Sink vs source limitation to starch biosynthesis

Maximum photosynthetic productivity of plants probably does not constitute a major limitation to starch accumulation in cereal grains (for reviews see Yoshida, 1972; Jenner, 1982). This is especially true for wheat, where there is no correlation of external CO_2 concentration with starch content of grains (Apel, 1976). In wheat also, Puckridge (1968) has shown that complete defoliation of plants plus ear shading does not lead to a significant reduction in grain yield (cited in Yoshida, 1972). More recently, P. Keeling and co-workers (I.C.I. Plant Biotechnology Group, Runcorn, Cheshire, U.K.; personal communications) have measured the flux of carbon from sucrose to starch in isolated wheat endosperm supplied with radiolabelled sucrose. Measurements of the intracellular sucrose concentration indicate that there is sufficient sucrose to fully saturate the starch biosynthetic pathway, indicating that starch biosynthesis in wheat endosperm is not source-limited. Thus, it is more likely that factors within the sink organ itself, such as a rate-limiting enzyme in the biosynthetic pathway from sucrose to starch, are the major limitations on starch biosynthesis in wheat endosperm.

2. The role of allosteric regulation in endosperm starch biosynthesis

At present there is no substantial evidence that post-translational regulation of ADP-glucose pyrophosphorylase operates *in vivo*, in non-photosynthetic plant organs. Table I.1 indicates that the ADP-glucose pyrophosphorylase enzyme from potato tuber has similar allosteric properties *in vitro* , to the enzyme isolated from plant leaves (see also Sowokinos and Preiss, 1982). However, potato tuber amyloplasts may differentiate into chloroplasts given the correct environmental stimulus, and it therefore possible that the starch biosynthetic pathway in potato tubers might resemble the pathway in leaves. If so, then we would also expect that similar regulatory mechanisms would operate in leaf chloroplasts and potato tuber amyloplasts. In contrast, the amyloplasts of cereal grains do not develop into chloroplasts during normal development and are functionally unrelated to chloroplasts. This fact, coupled with the nature of the starch biosynthetic pathway in wheat endosperm (see Section I.3.A. and Figure I.2) suggests that there may be no role for allosteric regulation of ADP-glucose pyrophosphorylase in cereal endosperm amyloplasts.

In support of this contention, P. Fentem and L. Catt (I.C.I. Plant Biotechnology Group, Runcorn, Cheshire, U.K.; personal communications) have studied the kinetics of partially-purified wheat endosperm ADP-glucose pyrophosphorylase. The results of that study (Table I.1.) demonstrate that the enzyme is not activated by concentrations of up to 20 mM 3-phosphoglycerate. The enzyme was also found to be only slightly inhibited by orthophosphate, with 700 μM orthophosphate required to achieve half-maximal inhibition of enzyme activity. This is much more than the concentration required to inhibit wheat leaf ADP-glucose pyrophosphorylase. A similar lack of allosteric responsivenes of maize endosperm ADP-glucose pyrophosphorylase was observed by Dickinson and Preiss (1969). In maize endosperm, this phenomenon has since been attributed to the effect of protease activity on the enzyme, since Plaxton and Preiss (1987) have demonstrated that maize endosperm ADP-glucose pyrophosphorylase is activated *in vitro* by 3-phosphoglycerate, when purified in the presence of 1.5 mM phenyl methane sulfonyl fluoride and 10 μg/ml chymostatin. However, in the case of the wheat endosperm ADP-glucose pyrophosphorylase enzyme, Fentem and Catt (personal communications) have also carried out the partial purification of enzyme activity in the presence of the protease inhibitors chymostatin, leupeptin and phenyl methane sulfonyl fluoride, and found no allosteric activation of that enzyme preparation by 3-phosphoglycerate. Thus, while we cannot rule out the possible effects of other proteases which are not inhibited by these chemicals, the results suggest that the wheat endosperm enzyme is not allosterically activated by 3-phosphoglycerate.

Consistent with the opposing effects of 3-phosphoglycerate and orthophosphate on leaf ADP-glucose pyrophosphorylase enzymes, inhibition of the wheat endosperm enzyme by orthophosphate is relieved by 3-phosphoglycerate. In the presence of 1 mM

3-phosphoglycerate, the $I_{0.5}$ value of wheat endosperm ADP-glucose pyrophosphorylase for orthophosphate, is increased to 1.5 mM. This indicates that although the enzyme cannot undergo the conformational changes necessary to convert it to a more active form in the presence of 3-phosphoglycerate, it is able to bind that metabolite. In view of this interaction of 3-phosphoglycerate and orthophosphate, the binding site for 3-phosphoglycerate is possibly close to the site for orthophosphate binding in the 3-dimensional structure of the enzyme.

The ADP-glucose pyrophosphorylase enzyme from rice endosperm is activated only 1.2-fold by 3-phosphoglycerate, with 5 mM 3-phosphoglycerate required to achieve half-maximal activation (Lee and Wang,. 1985; Table I.1). The maize endosperm enzyme is activated 20-fold by 3-phosphoglycerate (Plaxton and Preiss, 1987). However, this enzyme exhibits rather unique inhibition properties since the activator is required to achieve substantial inhibition by orthophosphate (Plaxton and Preiss, 1987; Table I.1). In the absence of 3-phosphoglycerate, a concentration of 10 mM orthophosphate inhibits the maize endosperm ADP-glucose pyrophosphorylase enzyme by only 10-20%; at 1 mM 3-phosphoglycerate concentration, the enzyme is completely inhibited by 8 mM orthophosphate ($I_{0.5}$= 440 μM). The variety of *in vitro* allosteric responses observed for the ADP-glucose pyrophosphorylases from rice, wheat and maize endosperm suggests that there may be few functional constraints on the maintenance of allosteric sites in these enzymes.

1. Identification of enzymes controlling the rate of endosperm starch biosynthesis

The most important regulatory feature of starch biosynthesis in the developing endosperm is probably a coarse control mechanism operating via regulation of the synthesis of starch biosynthetic enzymes. There has been considerable emphasis in recent research on the identification of rate-limiting enzymes in the starch biosynthetic pathway. Unfortunately, studies measuring the *in vitro* enzyme activities of starch biosynthetic enzymes throughout cereal endosperm development have not shed much light on this problem, since it is difficult to correlate *in vitro* and *in vivo* enzyme activities without knowledge of substrate pool sizes and concentrations of effector molecules, etc. In addition, genes coding for most of the starch biosynthetic enzymes appear to be expressed co-ordinately (Ozbun *et al*, 1973; Perez *et al*, 1975; Sowokinos, 1976; Tsai *et al*, 1970).

Research on maize genetics has identified several mutations in enzymes of the starch biosynthetic pathway which might prove useful in the identification of a rate-limiting enzyme. However, in order for these mutations to pinpoint an enzyme limiting the rate of starch biosynthesis *in vivo*, it is necessary to correlate dosage of mutant alleles at a particular locus with a particular enzyme deficiency and also with starch content. The enzyme deficiencies have been characterised for the *sh*-1 (Chourey and Nelson, 1976), *sh*-2 (Tsai and Nelson, 1966), *bt*-2 (Tsai and Nelson, 1966), *waxy* (*wx*; Boyer *et al*, 1978) and *amylose-extender* (*ae*; Boyer *et al*, 1978) mutations. Of these mutations, only the *sh*-2, *bt*-2, and *sh*-1 mutations significantly alter the total starch content of endosperm.

The *sh*-2 and *bt*-2 loci in maize code for the enzyme ADP-glucose pyrophosphorylase. In mutants homozygous for either or both mutant alleles, there is only 3-5% of the enzyme activity present in normal endosperms; increasing the numbers of functional alleles at both loci progressively increases enzyme activity in the endosperm (Hannah and Nelson, 1975). Although mutations in either the *sh*-2 or *bt*-2 loci drastically reduce endosperm starch content (Tsai and Nelson, 1966), the effect of gene dosage on starch content has not been reported. Gene dosage studies on *sh*-2 and *bt*-2 mutations are currently under being carried out by Chang and co-workers (Slater Research Centre, Garst Seed Company, Slater, IA50244, U.S.A.; personal communications). Thus, it is still possible that ADP-glucose pyrophosphorylase plays a role in regulating endosperm starch biosynthesis.

Chourey and Nelson (1976) have shown that the *sh*-1 locus codes for sucrose synthase. In maize lines homozygous for recessive mutations at this locus, the endosperm contains less than 10% of the sucrose synthase activity of normal endosperms. Despite such a huge decline in enzyme activity, the total starch content of mutant endosperms is reduced by only 30% compared to normal endosperms. It therefore seems that sucrose synthase is an unlikely candidate for limiting the rate of starch formation in maize endosperm.

There are several problems with the use of mutants in gene-dosage studies to isolate control points of biochemical pathways. Firstly, for any series of mutations which one wishes to analyse, it is necessary to produce mutations in an isogenic background, to avoid the contributing effects of other genes. Even when this is achieved, it is always possible that in the mutant plant the activities of other enzymes will compensate for the deficiencies produced by the mutation. As a result, the pathway may not be limited by the same factors in wild-type and mutant plants. Secondly, it is not always possible to obtain mutants with lesions in particular genes of interest. For example, there are no mutants available in which the activities of soluble starch synthase and UDP-glucose pyrophosphorylase are affected. In addition, there are no wheat mutants available which carry lesions in endosperm-specific starch biosynthetic enzymes. As a result, the application of such analytical methods is currently very limited. However, it might be possible in the near future to produce transgenic cereals which carry additional copies of starch biosynthetic genes. For example, a gene-dosage series might be constructed in wheat, with plants carrying 1,2,3,etc additional copies of the ADP-glucose pyrophosphorylase gene. The change in carbon flux from sucrose to starch resulting from increased ADP-glucose pyrophosphorylase activity could be measured to determine the sensitivity of the flux to changes in concentrations of this enzyme. If ADP-glucose pyrophosphorylase contributes to the net regulation of starch biosynthesis in the endosperm of the wild-type plant, then the level of enzyme activity and starch in the endosperm should increase in proportion to the extra copy number until the system is saturated. This experimental system would fulfill the requirement for an isogenic background in gene-dosage analysis. Providing that the regeneration of transformed monocot plants becomes a reality, then a molecular genetics approach to the problem of identifying rate-limiting enzymes in biochemical pathways will be a valuable technique.

As mentioned previously, the control of flux of metabolites through any biochemical pathway is probably shared between many enzymes, although under a predetermined set of conditions the contributions of individual enzymes to the overall control of flux is different (Kacser and Burns, 1973). The regulation of sucrose biosynthesis is given as an example of shared control in Section I.2.B.3. Another example of shared regulation is the control of gluconeogenesis in rat liver cells (Groen *et al*, 1986). If the glycolytic enzyme pyruvate kinase is inactive, as occurs when glucagon is present in the liver, then the flux from lactate to glucose is largely controlled by pyruvate carboxylase; however, in the absence of glucagon the control of gluconeogenesis is shared between pyruvate carboxylase, pyruvate kinase and fructose-1,6-bisphosphatase (Groen *et al*, 1986). Thus, it is important not to analyse any one enzyme of a biochemical pathway in isolation, but to determine the sensitivity of the flux to changes in the concentration of each enzyme of the pathway. In this way the relative contributions of each enzyme to the overall control of flux can be assessed.

4. FUNCTIONAL PROTEIN DOMAINS OF ADP-GLUCOSE PYROPHOSPHORYLASE

The characterisation of functional protein domains of regulatory enzymes is an important task, since it provides a basis on which we may assess the contribution of specific amino acid residues to various functions, for example substrate binding, allosteric effector binding, co-factor binding, etc. With this knowledge, protein engineering of functional domains becomes feasible. Several studies have been carried out on the ADP-glucose pyrophosphorylase enzyme from *Escherichia coli*, and more recently on the enzyme from spinach leaf, which help us to understand the regulatory properties of this enzyme. This section deals with the identification of substrate binding sites and allosteric effector binding sites of ADP-glucose pyrophosphorylase.

A. Substrate binding sites

Identification of the substrate binding site of *E.coli* ADP-glucose pyrophosphorylase has been performed by the covalent attachment of the photoaffinity analogue, 8-azido-ADP-[U-^{14}C]glucose to the enzyme (Lee and Preiss, 1986). Following labelling of the enzyme, it was digested with trypsin and the ^{14}C-labelled peptides were purified and sequenced. Photoaffinity labelling of the substrate binding site of *E.coli* ADP-glucose pyrophosphorylase results in a loss of enzyme activity (Parsons and Preiss,1978a; Lee and Preiss, 1986), presumably because the binding of additional substrate molecules to the active site is sterically hindered. In addition, the substrate affinity analogue is a competitive inhibitor with respect to ADP-glucose. Unlabelled ADP-glucose protects the enzyme 100% from photoinactivation by 8-azido-ADP-[U-^{14}C]glucose (Lee and Preiss, 1986), suggesting that the analogue does in fact bind to the ADP-glucose binding site. Two major peptide segments have been identified on the enzyme, which account for a total of 85% of the incorporated radioactivity (Figure I.3). Approximately 65% of the total incorporated radioactivity was found associated with the region between

Peptide segment 1 :

107 110
Met-Lys-Gly- Glu- Asn- Trp -Tyr -Arg- Gly-Thr-Ala-

120 128
Asp-Ala-Val- Thr- Gln -Asn- Leu -Asp-Ile-Ile-Arg

Peptide segment 2 :

162 170
Cys- Thr-Val -Val-Cys-Met- Pro -Val-Pro-Ile-Glu-Glu- Ala -Ser-

180
Ala-Phe-Gly -Val- Met -Ala- Val- Asp-Glu -Asn-Asp-Lys-Thr-Ile-

190 * 200
Glu-Phe -Val- Glu-Lys- Pro -Ala-Asn-Pro- Pro -Ser -Met -Pro-Asn-

207
Asp-Pro -Ser -Lys

Figure I.3 : Sequences of *E.coli* ADP-glucose pyrophosphorylase tryptic digests incorporating 8-azido-ADP- [U-^{14}C] glucose. Data taken from Lee and Preiss (1986). Residues which are conserved between *E. coli* and rice endosperm are boxed. Numbers refer to the relative position of residues in the *E. coli* ADP-glucose pyrophosphorylase enzyme. The peptide segment which is also involved in binding 8-azido- [α-^{32}P] ATP is underlined. Peptide segment 1 also binds the allosteric inhibitor analogue, [2-^{3}H] 8-azido-AMP (Larsen *et al*, 1986). Lys-194, which binds the allosteric activator, pyridoxal-5'-phosphate(Parsons and Preiss, 1978b), is also indicated (*).

residues 107-128 (peptide segment 1, Figure I.3), while only 20% of label was associated with the region between residues 162-207 (peptide segment 2, Figure I.3). Comparison of both peptide segments with the deduced partial amino acid sequence of rice endosperm ADP-glucose pyrophosphorylase (Preiss *et al*, 1987), reveals highly conserved domains which may be important in the binding of ADP-glucose (see Figure I.3). In the native enzyme, hydrolysis by trypsin occurs at Lys-108 and Arg-114 (peptide segment 1, Figure I.3) but cleavage at these positions is prevented by photoaffinity labelling of the enzyme, suggesting that the substrate analogue binds in the vicinity of these residues. The amino acid sequence adjacent to Arg-114 is also highly conserved between the *E.coli* and rice endosperm enzymes.

The substrate analogue 8-azido-[α-^{32}P]ATP binds the *E.coli* enzyme in the region between residues 115-128 (Lee and Preiss, 1986). This region is contained within the peptide segment incorporating the majority of labelled 8-azido-ADP-[U-^{14}C]glucose (i.e. peptide segment 1, Figure I.3), suggesting that the substrate binding sites for ATP and ADP-glucose overlap. In support of this suggestion, Lee and Preiss (1986) have also demonstrated that ATP inhibits by 70%, the labelling of the enzyme by 8-azido-ADP-[U-^{14}C]glucose.

B. Activator and inhibitor binding sites

ADP-glucose pyrophosphorylases can be classified into different groups on the basis of the metabolites that are most effective activators and inhibitors of the enzyme. The Enterobacteria accumulating glycogen, of which *E.coli* is an example, contain an enzyme which is activated primarily by fructose-1,6-bisphosphate and pyridoxal-5'-phosphate (Preiss, 1973). Secondary activators of the *E.coli* enzyme are 2-phosphoglycerate and phosphoenol pyruvate, while 3-phosphoglycerate does not activate the *E.coli* enzyme. As discussed in Section I.2.B.1., leaf ADP-glucose pyrophosphorylase enzymes are activated *in vitro* primarily by 3-phosphoglycerate and to a lesser extent by fructose-1,6-bisphosphate. In contrast, wheat endosperm ADP-glucose pyrophosphorylase is not activated by any of these metabolites (see Section I.3.B.2.). In addition, the *E.coli* enzyme is strongly inhibited by 5'-AMP and only weakly inhibited by orthophosphate, while all plant enzymes examined to date are strongly inhibited by orthophosphate (Preiss, 1973; Preiss and Walsh, 1981). It seems reasonable to assume that the different affinities of the various enzymes for fructose-1,6-bisphosphate, 3-phosphoglycerate and orthophosphate might be reflected in their primary structures.

Parsons and Preiss (1978a, 1978b) have covalently incorporated [^{3}H]pyridoxal-5'-phosphate into ADP-glucose pyrophosphorylase purified from *E.coli* mutant strain AC70R1, which contains depressed levels of the enzyme. The incorporation of this allosteric activator produces a modified enzyme of high specific activity, in the absence of additional fructose-1,6-bisphosphate (Parsons and Preiss, 1978a). Incorporation of [^{3}H]pyridoxal-5'-phosphate into the activator binding site is prevented by incubation of the enzyme in the presence of either fructose-1,6-bisphosphate or 5'-AMP, suggesting that the inhibitor and activator binding sites overlap in the tertiary structure of the enzyme. Subsequent trypsin digestion of the labelled enzyme, followed by peptide sequence analysis of the peptides generated, has been carried out by Parsons and Preiss (1978b). The allosteric activator binding site of *E.coli* ADP-glucose pyrophosphorylase is located at the N-terminus of the protein and the primary sequence of this peptide is shown in Figure I.4. Parsons and Preiss (1978b) have speculated that a preponderance of basic amino acids at the activator binding site, in addition to Lys-38 which binds [^{3}H]pyridoxal-5'-phosphate, is required for the binding of other activators such as fructose-1,6-bisphosphate. At present, there are no amino acid sequence data covering the corresponding region of a plant ADP-glucose pyrophosphorylase enzyme with which to compare this binding site.

Interestingly, Parsons and Preiss (1978b) also demonstrated that [^{3}H]pyridoxal-5'-phosphate binds to Lys-194, which is within the peptide segment observed to bind 20% of the photoaffinity analogue, 8-azido-ADP-[U-^{14}C]glucose (Lee and Preiss, 1986; see peptide segment 2, Figure I.3). Incorporation of the allosteric activator at this site is inhibited by ADP-glucose plus $MgCl_2$ (Parsons and Preiss, 1978a), indicating that the substrate binding site overlaps with an activator binding site in the 3-dimensional structure of the native enzyme.

The 3-phosphoglycerate binding site of the spinach leaf ADP-glucose pyrophosphorylase enzyme has been elucidated (Preiss *et al*, 1987; J. Preiss, Dept Biochemistry, Michigan State University, East Lansing, Michigan, U.S.A.; personal communications). Pyridoxal-5'-phosphate is a weak allosteric activator of this enzyme compared to 3-phosphoglycerate, since the maximum stimulation achieved with pyridoxal-5'-phosphate is only 5- to 6-fold (Preiss *et al*, 1987). However, this metabolite can

10
NH_2 – Val – Ser – Leu – Glu – Lys – Asn – Asp – His – Leu – Met –

20
Leu – Ala – Arg – Gln – Leu – Pro – Leu – Lys – Ser – Val –

30
Ala – Leu – Ile – Leu – Ala – Gly – Gly – Arg – Gly – Thr –

40
Arg – Leu – Lys – Asp – Leu – Thr – Asn – $\overset{*}{\text{Lys}}$ – Arg – Ala –

50
Arg – Pro – Ala – Val – His – Phe – Gly – Gly – Lys – Phe –

60
Arg – Ile – Ile – Asp – Phe – Ala – Leu – Ser – Asn – Cys –

68
Ile – Asn – Ser – Gly – Ile – Arg – Arg – Met

Figure I.4 : N-terminal sequence of *E. coli* ADP-glucose pyrophosphorylase. Residues 1-44 incorporate the allosteric activator, [^{3}H] pyridoxal-5'-phosphate (Parsons and Preiss, 1978b). The lysine residue involved in binding pyridoxal-5'-phosphate is indicated (*). Residues 11-68 have been shown to bind the allosteric inhibitor analogue, [2-^{3}H] 8-azido-AMP (Larsen *et al*, 1986). Numbers refer to the relative position of amino acid residues in the *E. coli* enzyme.

inhibit the activation caused by 3-phosphoglycerate and reduce the sensitivity of the enzyme to orthophosphate, suggesting that the binding site for pyridoxal-5'-phosphate and 3-phosphoglycerate may be shared. The amino acid sequence of the allosteric activator binding site has been determined (J. Preiss, personal communications) and is presented in Figure I.5. The sequence is well-conserved between spinach leaf and rice endosperm amino acid sequences, suggesting that both enzymes might equally be capable of binding pyridoxal-5'-phosphate (and 3-phosphoglycerate), despite the very different *in vitro* allosteric responses of these enzymes to the metabolite. Further sequence comparisons of leaf and endosperm ADP-glucose pyrophosphorylase enzymes are required to obtain a consensus sequence for the allosteric activator binding site of plant ADP-glucose pyrophosphorylases.

In addition to the studies mentioned above, three 5'-AMP binding sites on the *E.coli* ADP-glucose pyrophosphorylase enzyme have recently been elucidated by covalent linkage of the photoaffinity inhibitor analogue [2-^{3}H]8-azido-AMP to the enzyme (Larsen *et al*, 1986). These authors have shown that the major binding site for the analogue overlaps with the ATP and ADP-glucose binding site located between residues 107-128 of *E.coli* ADP-glucose pyrophosphorylase (peptide segment 1, Figure I.3). The second inhibitor binding site identified is located between residues 11-68 of the *E.coli* enzyme, which overlaps with the allosteric activator binding site containing Lys-38 (Figure I.4). The third inhibitor binding region identified incorporates only 15% of the [2-^{3}H]8-azido-AMP and it is located at residues 222-254 (Larsen *et al*, 1986). The amino acid sequence of this third 5'-AMP binding site is presented in Figure I.6. Since it has

Ser – Gly – Ile – Val – Thr – Val – Ile – $\overset{*}{\text{Lys}}$ – Asp – Ala – Leu – Pro – Ser

Figure I.5 : Sequence of the spinach leaf ADP-glucose pyrophosphorylase tryptic peptide incorporating $[^3H]$ pyridoxal-5'-phosphate. The residue involved in binding pyridoxal-5'-phosphate is indicated (*). Data provided by J. Preiss (Dept biochemistry, Michigan State University, East Lansing, Michigan, U.S.A.; personal communications). The peptide segment is located near the C-terminus of the protein, but the exact position is unknown.

222 … 230
Leu – Tyr – Glu – Leu – Leu – Glu – Glu – Asp – Asp – Arg –

240
Asp – Glu – Asn – Ser – Ser – His – Asp – Phe – Gly – Lys –

250
Asp – Leu – Ile – Pro – Lys – Ile – Thr – Glu – Ala – Gly –

254
Leu – Ala – Tyr

Figure I.6 : Sequence of the minor [2-^{3}H] 8-azido-AMP labelled tryptic peptide of *E. coli* ADP-glucose pyrophosphorylase. Data from Larsen *et al.* (1986). Numbers refer to the position of amino acid residues in the *E. coli* protein.

not been demonstrated that the binding of the 5'-AMP analogue is inhibited by orthophosphate, we cannot be certain whether these inhibitor binding sites will be functional in the binding of orthophosphate to plant ADP-glucose pyrophosphorylase enzymes. Given the different inhibitor preferences of the *E.coli* and plant enzymes, this suggestion seems unlikely. We must therefore await the elucidation of orthophosphate binding sites on plant ADP-gluose pyrophosphorylase enzymes.

5. AIM OF THE PRESENT WORK

The overall aim of the work described in this thesis was to determine whether the observed differences in allosteric properties of wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase activities are the result of organ-specific gene expression. In the first part of this work, it was intended that the ADP-glucose pyrophosphorylase activity would be purified from developing wheat endosperm. It was envisaged that, in addition to providing information on the subunit structure and N-terminal amino acid sequence of the endosperm enzyme, the purified protein would be injected into rabbits for the preparation of a specific immune serum. Western-blot analysis of wheat endosperm and wheat leaf soluble protein would follow, as a means of comparing the subunit structures of the wheat endosperm and wheat leaf ADP-glucose pyrophosphorylase enzymes.

A subsequent part of the study was the isolation and characterisation of cDNA clones encoding the wheat endosperm and wheat leaf ADP-glucose pyrophosphorylases. These cDNAs were to be used initially in hybridisation analyses to compare the organisation of genes and sizes of mRNA transcripts encoding both ADP-glucose pyrophosphorylase isoenzymes. Determination of the nucleotide sequences and derived amino acid sequences of wheat leaf and endosperm ADP-glucose pyrophosphorylases would follow the gene organisation studies. The identification of the primary translation products of wheat endosperm and wheat leaf ADP-glucose pyrophosphorylase mRNAs was considered to be important in this analysis; it was envisaged that a comparison of the sizes of nascent and mature polypeptides, coupled with nucleotide sequence analysis of full-length cDNA clones, would assist in determining whether different transit peptide sequences are involved in the transport of proteins to chloroplasts and amyloplasts. It was of particular importance to identify amino acid sequences in the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase isoenzymes which are related to the substrate, inhibitor and activator binding sites of other ADP-glucose pyrophosphorylase enzymes. A comparison of these sequences between wheat leaf and wheat endosperm isoenzymes will be of considerable use in helping us to understand the different kinetics of these enzymes.

SECTION II- MATERIALS AND METHODS

1. MATERIALS

A. Chemicals, biochemicals, radiochemicals and enzymes

All materials used were of the highest analytical grade available. The source of specific materials is shown below under the supplier's name.

Aldrich Chemical Co Ltd, Gillingham, Dorset: Methyl sulfoxide, HPLC grade (DMSO)

Amersham International plc, Amersham, Buckinghamshire: Adenosine 5'-[γ-^{32}P]triphosphate (3000 Ci/mmol); amino acid mixture minus methionine (1μM each amino acid); 2'- deoxyadenosine 5'-[α-^{35}S]thiotriphosphate (>1000 Ci/mmol); 2'-deoxyadenosine 5'-[α-^{32}P]triphosphate (>400 Ci/mmol); 2'-deoxycytidine 5'-[α-^{32}P]triphosphate (>400 Ci/mmol); cDNA synthesis system, α-D-[U-^{14}C]glucose-1-phosphate (>150 mCi/mmol); Hybond-N nylon blotting membranes; L-[^{35}S]methionine (>800 Ci/mmol); [^{14}C] methylated protein molecular weight markers for gel electrophoresis (10-50 μCi/mg protein); T_4 polynucleotide kinase; Protein A, ^{125}I-labelled with Bolton and Hunter reagent, affinity purified (>30 mCi/ mg protein); Rabbit Ig, ^{125}I-labelled whole antibody (from donkey); Rabbit reticulocyte lysate, amino acid depleted; Restriction enzyme buffers (sterile 10X stocks).

Analytichem International, Harbor City, CA90710, U.S.A: Bond Elut SAX Extraction Cartridges.

BDH Chemicals Ltd, Poole, Dorset: Acetic acid; acetone; acrylamide, Grade 1 'Electran'; ammonia; ammonium acetate; boric acid; bromophenol blue, 'Electran'; chloroform; di-potassium hydrogen orthophosphate; ethylenediamine tetra-acetic acid; Folin and Ciocalteu's phenol reagent; D-glucose; N-glycylglycine; hydrochloric acid; hydrogen peroxide (100 volume); N-2-hydoxyethylpiperazine-N'-2-ethanesulphonic acid; magnesium chloride hexahydrate; methanol; N,N'-methylenebisacrylamide,Grade 1 'Electran'; PAGE blue 83, 'Electran'; phenylmethane sulphonyl fluoride; polyethylene glycol$_{6000}$ (PEG$_{6000}$); potassium acetate; potassium chloride; potassium dihydrogen orthophosphate; potassium tartrate; sodium acetate; sodium chloride, sodium dodecyl sulphate; sodium hydroxide; sodium nitrite; N,N,N',N'-tetra methylethylenediamine, Grade 1 'Electran'; Tris (hydroxymethyl)methylamine; urea.

Bethesda Research Laboratories (U.K.) Ltd, Cambridge, Cambridgeshire: 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal); dithiothreitol; deoxyribonuclease (ribonuclease-free); formamide; glycerol; isopropylthio-β-galactoside (IPTG); Nick translation reagent kit; Nonidet P40; phenol, redistilled nucleic acid grade; proteinase K; ribonuclease (deoxyribonuclease-free); sucrose (nuclease-free).

Bio-Rad Laboratories Ltd, Watford, Hertfordshire: Bio-gel A150 (100-200 mesh); mixed bed resin AG501-X8 (20-50 mesh).

Bio-Yeda Ltd, Kiryat Weizmann, Rehovot, Israel: Horseradish peroxidase conjugated anti-rabbit IgG, from goat.

Boehringer Corporation (London) Ltd, Lewes, Sussex: Adenosine-5'-triphosphate; albumin, fraction V from bovine serum; alkaline phosphatase from calf intestine, molecular biology quality; di-ammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS); glucose GOD-Perid assay kit; glucose-6-phosphate dehydrogenase; Klenow fragment of DNA polymerase; oxaloacetic acid; phosphoglycerate mutase; transfer ribonucleic acid (tRNA).

Calbiochem Biochemicals, San Diego, California, U.S.A: Lysozyme, egg white.

Collaborative Research Inc, Bedford, MA 01730, U.S.A: Oligo (dT)-cellulose Type 2.

Difco Laboratories Ltd, Detroit, Michigan, U.S.A: Bactoagar; bactotryptone; trypticase; yeast extract.

Fisons Scientific Apparatus, Loughborough, Leicestershire: Caesium chloride.

Fluka AG, Buchs, Switzerland: Glyoxal.

New England Biolabs, Beverly, MA 01915-9990, U.S.A: *Eco*RI methylase; s-adenosylmethionine.

NEN Research Products, Stevenage, Hertfordshire: EN^{3}HANCE autoradiography enhancer; GeneScreen Plus nylon blotting membrane.

Oxoid Ltd, Basingstoke, Hants: Phosphate-buffered saline (Dulbecco'A').

Pharmacia Fine Chemicals, Uppsala, Sweden: Agarose; dextran sulphate; *Eco*RI linkers [5'-d(GGAATTCC)-3']; high molecular weight gel filtration calibration kit; lambda DNA-*Hind*III digest, DNA size markers; low molecular weight gel electrophoresis calibration kit; methyl-7-guanosine cap analogue [$m^7G(5')ppp(5')G$]; mono Q HR 5/5 ; prepacked PD10 columns; protein A sepharose 6MB; restriction enzymes; sephadex G-50 fine; sepharose CL-4B; single-strand binding protein (*Escherichia coli*); superose HR 10/30; T_4 DNA ligase, FPLC pure; T_4 RNA ligase (RNase and DNase free, from T_4-infected *E.coli*).

Schleicher and Schuell, Dassel, West Germany: BA 85/20 nitrocellulose filter discs.

Sigma Chemical Company Ltd, Poole, Dorset: Adenosine 5'-diphosphoglucose; adenosine 2' and 3'-monophosphoric acid; ampicillin; deoxycholic acid; ethidium bromide; ficoll (molecular weight 400,000); α-D-glucose-1,6-diphosphate; glutathione; heparin (from *Porcine sp.* intestinal mucosa); hexadecyltrimethylammonium bromide (CTAB); imidazole; β-mercaptoethanol; α-nicotinamide adenine dinucleotide, reduced form; β- nicotinamide adenine dinucleotide 3'-phosphate; octyl phenoxy polyethoxyethanol (Triton X-100); *o*-phenylenediamine; polyoxyethylene sorbitan monolaurate (Tween 20); polyvinyl pyrrolidone; sodium azide; sodium deoxycholate; trichloroacetic acid; urea hydrogen peroxide.

Stratagene Cloning Systems,(U.K. supplier NBL Ltd, Cramlington, Northumberland): DSK-[^{35}S]-deoxynucleotide 5'-triphosphate dideoxy sequencing system; gigapack gold *in vitro* packaging kit; *in vitro* transcription kit.

B. Bacterial, plasmid and bacteriophage stocks

Bacteria, plasmid and bacteriophage stocks are shown in Table II.1 with the genotype, literature reference and supplier.

C.Plant material

Wheat (*Triticum aestivum* c.v. Mardler) seeds were obtained from the National Seed Development Organisation, Cambridge.

2. GROWTH OF PLANTS

Winter wheat (*Triticum aestivum* c.v. Mardler) seeds were sown in John Innes compost No3 at a density of 50 seeds per 15 cm diameter pot and kept in a glasshouse until shoots appeared. Glasshouse temperature was maintained between 15°C maximum during the day and 5°C minimum during the night. Seedlings were then transferred to a chill cabinet for 5 weeks with temperature maintained at 8°C±2°C and a 16 hr photoperiod (light intensity during the day kept at 150-200 μEm^2s^{-1} supplied by a combination of fluorescent and tungsten lights). The plants were subsequently

Table II.1 : Sources of bacterial, plasmid and bacteriophage stocks

Stock	Genotype	Reference	Supplier
E. coli TG-2	rec A13, r_k^-, m_k^+, Δ lacpro, thi^-, supE, F', tra D36, proAB, lac I^qZ(M15)	A. Bankier, personal communications, MRC Laboratory, Cambridge	Dr C. Bird ICI Runcorn.
E. coli Y1088	Δ lacU169, supE, supF, $hsdR^-$, $hsdM^+$, metB, trpR, tonA21, proC::Tn5 (pMC9), pMC9=pBR322- $lacI^q$	Young and Davies (1983b)	Stratagene
E. coli Y1090	Δ lacU169, $proA^+$, Δ lon, araD139, strA, supF [trpC22::Tn10] (pMC9)	Young and Davis (1983b)	Stratagene
Plasmid pBSM13+	$lacI^qZ$, Amp^r,	Stratagene	Stratagene
Lambda gt11	lac5, c1857, nin5, S100	Young and Davis (1983a)	Stratagene

transplanted into fresh compost at a density of 1 plant per pot and grown for 4 weeks longer in a controlled environment cabinet. The temperature during the light cycle was 15°C and during the dark cycle was 10°C. The photoperiod and light intensity were as previously stated. Relative humidity was maintained at 80%. Finally, plants were removed to another cabinet maintained at 20°C during the light cycle and 15°C during the dark cycle. Approximately 3-4 months after germination, when the anthers first appeared, the ears were tagged to show the date of anthesis. The age of wheat endosperm was taken relative to the date of anthesis and recorded as days post anthesis (d.p.a.).

Plants were fed nutrient fertiliser Phostrogen (Phostrogen Ltd, Corwen, Clwyd) every 2 weeks and watered continuously by automatic bottom damping throughout the growth period. Plants were also sprayed every week with Milgoe (I.C.I. Plant Protection Division, Jealotts Hill, Berkshire) to control mildew and with Picket G (I.C.I. Plant Protection Division) every 2 weeks to control grain aphid pests.

For experiments requiring only wheat leaf tissue the seeds were sown at high density in seed trays and these kept for 7 days in a controlled environment maintained at 20°C during the light cycle and 15°C during the dark cycle. Light intensity, photoperiod and relative humidity were the same as for more mature plants (see above). If the object of the experiment was to extract high molecular weight DNA from seedlings, they were removed to a dark room 5 days after planting and left for 48 hr prior to harvesting the leaves.

3. ENZYME ASSAYS

A. ADP-glucose pyrophosphorylase

1. Forward reaction

ADP-glucose pyrophosphorylase activity was measured in the direction of ADP-glucose synthesis using a method modified from that of Shen and Preiss (1964). All steps were carried out at 4°C unless otherwise stated. For routine assays a crude homogenate was prepared by grinding endosperm tissue in 10 volumes (w/v) of 50 mM glycylglycine/NaOH pH 7.5, 0.1 mM DTT using a chilled mortar and pestle. The crude homogenate was clarified by centrifugation at 27,000 x g for 15 min. The supernatant was used as a source of enzyme activity.

Enzyme reactions were carried out in Eppendorf microcentrifuge tubes which contained components in the following final concentrations in a volume of 200 µl:

5% (v/v)	enzyme extract
100 mM	Hepes/NaOH, pH 8.0
5 mM	$MgCl_2$
4 mM	ATP
250 µg/ml	bovine serum albumin
0.05 units/ml	inorganic pyrophosphatase
0.35 µCi/ml	α-D-[u-^{14}C]glucose-1-phosphate (51.8 mCi/mmol)

Reactions were started by the addition of radioactively labelled glucose-1-phosphate. Control samples were boiled for 90 sec prior to the addition of substrate. Samples were incubated at 37°C for 5 min and the reactions were stopped by boiling samples for 90 sec.

The product of the reaction, ADP-[U-^{14}C]glucose, was separated from the labelled substrate by ion-exchange chromatography according to P. Keeling (I.C.I. Plant Biotechnology Group, Runcorn, Cheshire; personal communications). The reaction mixture was loaded onto a Bond Elut SAX cartridge. The column was washed with 3 ml distilled water followed by 40 ml of 80 mM formic acid to remove the substrate. The reaction product was then eluted in 3 ml of 500 mM ammonium formate and counted in 10 ml scintillant (Beckman MP) in an LKB Rackbeta scintillation counter.

ADP-glucose pyrophosphorylase activity was calculated from the specific activity of α-D-[U-^{14}C]glucose-1-phosphate used to start the reaction. Enzyme activity was expressed as 'nkatal/gm fresh weight', 'nkatal/endosperm', or 'nkatal/mg protein' where one nkatal is the enzyme activity required to convert one nmole glucose-1-phosphate to ADP-glucose in one second under the assay conditions described.

2. Reverse Reaction.

ADP-glucose pyrophosphorylase activity was also measured in the direction of pyrophosphorylysis by coupling this reaction to the reduction of NADP+ via the phosphoglucomutase and glucose-6-phosphate dehydrogenase reactions, essentially according to Sowokinos (1981). A tissue extract was prepared as described as described in Section II.3.A.1. Enzyme reactions were carried out in 1 ml disposable cuvettes and contained components in the following final concentrations in a volume of 1 ml:

1.5% (v/v)	enzyme extract
62.5 mM	glycylglycine/NaOH, pH 7.7
3.8 mM	$MgCl_2$
2.0 mM	ADP-glucose
0.6 mM	NADP+
0.02 mM	glucose-1,6-bisphosphate
1.2 unit/ml	glucose-6-phosphate dehydrogenase
3.4 unit/ml	phosphoglucomutase
1.5 mM	inorganic pyrophosphate

The absorbance was measured at 340 nm (30°C) for 2 min prior to the addition of the substrate, inorganic pyrophosphate, to obtain a baseline level of NADPH production. The reaction was then started by addition of substrate and the rate of change in absorbance measured.

ADP-glucose pyrophosphorylase activity was calculated from the molar extinction coefficient of NADPH at 340 nm (6.2×10^3 $M^{-1}cm^{-1}$). Enzyme activity was expressed in 'nkatal/gm fresh weight' or 'nkatal/mg protein'.

B. UDP-glucose pyrophosphorylase

UDP-glucose pyrophosphorylase activity was measured in the direction of pyrophosphorylysis as outlined in Section II.3.A.2

with the exception that ADP-glucose was replaced by 2 mM UDP-glucose in the reaction mixture.

C. Malate dehydrogenase

Malate dehydrogenase was measured in the direction of oxaloacetate reduction according to Bergmeyer (1981). A tissue extract was prepared as described in Section II.3.A.1. Enzyme reactions were carried out in 1 ml disposable cuvettes in a final volume of 1 ml. The reaction mixture contained components in the following final concentrations:

10%(v/v)	enzyme extract
30 mM	potassium phosphate buffer, pH 7.0
0.2 mM	NADH
3 mM	EDTA
0.5 mM	oxaloacetate

The absorbance was measured at 340 nm (30°C) for 2 min prior to the addition of oxaloacetate. The reaction was then started by the addition of substrate and the rate of change in absorbance was measured. Malate dehydrogenase activity was calculated using the molar extinction coefficient of NADH (6.2×10^3 $M^{-1}cm^{-1}$). Enzyme activity was expressed in 'nkatal/mg protein'.

4. DETERMINATION OF PROTEIN

Soluble protein was determined by the method of Peterson (1977) using bovine serum albumin (fraction V) for the calibration of standard curves. In a routine assay, the protein solution was made to a final volume of 1 ml with distilled water and 100 µl of 0.15% (w/v) sodium deoxycholate was added. After incubation of samples at room temperature for 10 min, protein was precipitated by addition of 100 µl of 72% (w/v) TCA. Protein was collected by centrifugation at 4,000 x g for 15 min and resuspended in 1 ml of distilled water. To the protein suspension was added 1 ml of Reagent A:

200 mM	NaOH
2.5% (w/v)	Na_2CO_3
0.05% (w/v)	potassium tartrate
0.025% (w/v)	$CuSO_4$
2.5% (w/v)	SDS

Following incubation at room temperature for 10 min, 500 µl of a 1/6 dilution of Folin and Ciocalteu's Reagent in water was added. The samples were mixed and incubated at room temperature for 30 min. The absorbance at 750 nm was determined for samples and protein standards.

5. DETERMINATION OF STARCH

The starch content of wheat endosperms was determined using the method of P. Keeling (I.C.I. Plant Biotechnology Group, Runcorn, Cheshire; personal communications). A crude homogenate was prepared as described in Section II.3.A.1. Following centrifugation as described, the starchy pellet was retained and washed three times with ice-cold distilled water. The starch was resuspended in 4 ml of 100 mM sodium acetate pH 4.5. This suspension was boiled for 45 min, cooled to room temperature and then 90 units of amyloglucosidase (α-1,4 and α-1,6-glucan hydrolase) from *Rhizopus* mould were added. Following incubation at 37°C for 24 hr, the samples were centrifuged at 4,000 x g for 10 min. The supernatants were assayed colorimetrically for glucose content using the Glucose-GOD-Perid assay kit according to Werner *et al* (1970). In a standard assay, 100 µl of either a standard glucose solution or the assay supernatant was added to 3 ml of enzyme/substrate solution:

100 mM	phosphate buffer, pH 7.0
≥0.8 units/ml	horseradish peroxidase
≥10.0 units/ml	glucose oxidase
1 mg/ml	ABTS

Solutions were mixed and incubated at 25°C for 30 min. The absorbance at 610 nm was measured for both assay samples and glucose standards. The starch content of endosperms was expressed in 'µmol glucose/ endosperm'.

6. PURIFICATION OF WHEAT ENDOSPERM ADP-GLUCOSE PYROPHOSPHORYLASE

A. Purification strategy

ADP- glucose pyrophosphorylase was required in pure form for the production of antibodies and also for N-terminal amino acid sequence analysis. The wheat endosperm ADP-glucose pyrophosphorylase enzyme proved to be difficult to purify to homogeneity using conventional chromatographic procedures. In pilot experiments the enzyme proved to be very labile when concentrated for gel filtration chromatography. In addition, the stability of the enzyme was maintained only in potassium phosphate buffer, therefore restricting the variety of chromatographic steps which could be included in a purification protocol. Because the enzyme was not necessarily required in an active form, it was decided to carry out a partial purification of enzyme activity using conventional liquid chromatographic procedures and then to purify putative ADP-glucose pyrophosphorylase polypeptides from SDS/polyacrylamide gels.

The columns used in the purification protocols were chosen after trials of several chromatographic matrices. Sepharose CL-6B proved to be unsuccessful because the enzyme activity eluted in the void volume. Results obtained using an affinity chromatography matrix, ATP-agarose (C8-AGATP), were highly variable.

Two protocols were employed for the partial purification of enzyme activity. Purification protocol 1, described in Section II.6.B was improved upon during the course of the project to produce purification protocol 2 (Section II.6.C), which allows for a more rapid purification of enzyme activity. In protocol 1, the first stage of the purification employed DEAE-Sepharose as an ion-exchange medium. Reproducible results were obtained; the resin was easy to pack into columns and allowed reasonably high flow rates. Although Sephacryl S-300 sf proved to be a suitable gel filtration medium, I chose to take advantage of the much higher flow rates and sharper resolution obtainable with the FPLC gel filtration medium Superose 12 HR 10/30.

In purification protocol 2, the first stage of the purification employed phenyl-Sepharose as a hydrophobic chromatography medium. The ion-exchange medium used was MonoQ HR 5/5 because of the high flow rates and sharp resolution obtainable with the FPLC system. The salt gradient used was determined from the results of experiments employing low pressure ion-exchange chromatography. A second purification of samples on MonoQ HR 5/5 was found to produce greater resolution of ADP-glucose pyrophosphorylase activity than was observed during the first chromatography on this medium. Gel filtration was carried out as described in purification protocol 1.

B. Partial purification of enzyme activity: Protocol 1

All steps were carried out at 4°C except for those employing FPLC which were at room temperature. Endosperm (5 g) was dissected from 21-day old wheat seeds and homogenised using a Polytron (low speed setting for 1 min) in 2 volumes (w/v) of homogenisation buffer:

30 mM	potassium phosphate, pH 7.0
0.5 mM	EDTA
5 mM	$MgCl_2$
0.1 mM	DTT

The extract was centrifuged at 39,000 x g for 20 min. The supernatant was retained as a source of enzyme and dialysed overnight in 2 dm^3 of 10 mM potassium phosphate pH 7.0, 0.1 mM glutathione.

Ion-exchange chromatography was carried out according to Fuchs and Smith (1979). The dialysed supernatant was adsorbed onto a DEAE-Sepharose column (1.0 cm x 6.6 cm) which had been previously equilibrated with 10 mM potassium phosphate pH 7.0, 0.1 mM glutathione. The column was then washed successively with 5 bed volumes each of 10 mM, 30 mM and 50 mM potassium phosphate pH 7.0, containing 0.1 mM glutathione. A linear gradient of 0-500 mM KCl in 50 mM potassium phosphate pH 7.0, 0.1 mM glutathione was applied to the column and fractions of 1 ml were collected. The flow rate was maintained at 0.3 ml/min throughout the procedure. Fractions were assayed for ADP-glucose pyrophosphorylase activity as described in Section II.3.A.1. The ultraviolet absorbance at 280 nm was recorded throughout the entire procedure. Fractions (x-2) to (x+2) (where x = ADP-glucose pyrophosphorylase peak fraction) were pooled, assayed for protein content as described in Section II.4 and finally concentrated by osmosis against PEG_{6000}.

This enzyme preparation was applied to a Superose 12 HR 10/30 column (1.5 cm x 30 cm) equilibrated previously with gel filtration buffer:

50 mM	potassium phosphate, pH 7.0
150 mM	KCl
0.1 mM	glutathione

The column was eluted at 0.5 ml/min using the same buffer. Fractions of 0.5 ml were collected and placed on ice. Fractions were assayed for ADP-glucose pyrophosphorylase activity as described in Section II.3.A.1. The ultraviolet absorbance at 280 nm was monitored continuously throughout the procedure. The two fractions containing maximum enzyme activity were pooled and dialysed overnight against 10 mM Tris/HCl pH 7.0. This protein preparation was stored at -70°C until sufficient samples were available for a large-scale purification of polypeptides from SDS/polyacrylamide gels (Section II.6.D).

C. Partial purification of enzyme activity: Protocol 2

All steps were carried out at 4°C except for FPLC operations which were at room temperature. Endosperm (5 g) was homogenised as described in Section II.6.B. To 20 ml of the supernatant, 18.8 ml of 1 M potassium phosphate pH 7.0 was added very slowly. The protein solution was applied to a 5 ml column of phenyl-Sepharose 4B (0.6 cm x 5 cm) equilibrated previously with 500 mM potassium phosphate pH 7.0. The column was washed with 20 ml of the same buffer and then with 20 ml of 350 mM potassium phosphate pH 7.0. ADP-glucose pyrophosphorylase activity was eluted with 10 mM potassium phosphate pH 7.0 and fractions of 1 ml volume were collected. The flow rate was maintained at 0.5 ml/min and the ultraviolet absorbance at 280 nm was continuously monitored throughout the procedure. ADP-glucose pyrophosphorylase activity of fractions was measured as described in Section II.3.A.2 and those containing enzyme activity were pooled. The enzyme preparation was desalted into 10 mM potassium phosphate pH 7.0, using prepacked PD10 columns as described by the manufacturer.

The desalted enzyme preparation was adsorbed onto MonoQ HR 5/5 previously equilibrated with 10 mM potassium phosphate pH 7.0. The column was washed with 8 ml of the same buffer and then a linear gradient of 0-350 mM KCl in 10 mM potassium phosphate pH 7.0 was applied. Fractions of 1 ml were collected. The ultraviolet absorbance at 280 nm was monitored continuously throughout the procedure. Fractions were assayed for ADP-glucose pyrophosphorylase activity as described in Section II.3.A.2 and fractions (x-2) to (x+3) (where x = ADP-glucose pyrophosphorylase peak fraction) were pooled. The enzyme preparation was subsequently desalted and rechromatographed on MonoQ HR 5/5 as before.

The eluant from the second round of purification on MonoQ HR 5/5 was concentrated and chromatographed on Superose 12 HR 10/30 as described in Section II.6.B. The partially purified ADP-glucose pyrophosphorylase preparation was dialysed overnight against 10 mM Tris/HCl pH 7.0 and stored at -70°C until sufficient samples were available for preparative gel electrophoresis (Section II.6D.)

D. Electroelution of protein from polyacrylamide gels

Partially purified wheat endosperm ADP-glucose pyrophosphorylase preparations were electrophoresed on SDS/polyacrylamide gels as described in Section II.7.A. Proteins were visualised by staining with PAGE blue 83 as described in Section II.7.B. For experiments involving the preparation of protein for N-terminal sequence analysis, the duration of staining with PAGE blue 83 was decreased to 10 min, as recommended by Walker *et al* (1982). Gel slices containing polypeptide bands of interest were excised from polyacrylamide gels using a scalpel blade. The gel slices were placed into a glass tube (1 cm x 9 cm) containing a porous polyethylene filter at the lower end. A piece of dialysis tubing (1 cm diameter) was placed over the lower end of the glass tube. The assembly was then filled from both open ends with elution buffer:

25 mM	Tris base
192 mM	glycine
0.1% (w/v)	SDS

pH 8.3

The open end of the dialysis tube was then sealed with a clamp and the assembly placed into a tube gel electrophoresis tank containing elution buffer. A current of 15 mA was applied for 20 hr, after which time the stained proteins had collected in the dialysis tubing. The dialysis tubing was then detached from the glass tube containing the gel slices. Samples required for antibody production were dialysed against 3 x 5 dm^3 of 10 mM Tris/HCl pH 7.0. Samples required for amino acid sequence analysis were dialysed extensively against sterile distilled water. Protein samples were lyophilised and stored at 4°C until required.

7. GEL ELECTROPHORESIS OF PROTEINS

A. SDS/polyacrylamide gel electrophoresis

Proteins were electrophoresed on SDS/polyacrylamide gradient slab gels employing the gel system of Laemmli (1970). Gels were a modification of the type used by J.E. Musgrove (Dept of Biological Sciences, University of Warwick, Coventry; personal communications). The resolving gel contained a linear gradient of 10-20% (w/v) acrylamide. Each acrylamide solution was prepared to give the following final concentrations of reagents:

Reagent	Solution A	Solution B
acrylamide	10% (w/v)	20% (w/v)
bisacrylamide	0.16% (w/v)	0.1% (w/v)
glycerol	-	30% (v/v)
Tris/HCl, pH 8.8	375 mM	375 mM
SDS	0.1% (w/v)	0.1% (w/v)
TEMED	0.03% (v/v)	0.04% (v/v)
ammonium persulphate	0.025% (w/v)	0.007% (w/v)

The total volume of the resolving gel was 30 ml per slab. The gradient was formed using a Bio-Rad Model 385 gradient former and pumped between two glass plates (18 cm x 16 cm) separated by a 0.15 cm thick spacer. The gradient was overlaid with distilled water containing a few drops of 10% (w/v) SDS and then allowed to polymerise.

The top of the resolving gel was then washed with distilled water prior to addition of the stacking gel. The stacking gel solution contained the following final concentrations of reagents:

0.5% (w/v)	acrylamide
0.25% (w/v)	bisacrylamide
240 mM	Tris/HCl, pH 6.8
0.1% (w/v)	SDS
0.05% (v/v)	TEMED
0.1% (w/v)	ammonium persulphate

The stacking gel mixture was pipetted between the glass plates onto the resolving gel and a teflon comb inserted into place. After polymerisation of the stacking gel, the comb was removed and the loading wells were washed with distilled water to remove any unpolymerised acrylamide. The gel was then mounted in the electrophoresis tank (Hoefer Scientific Instruments, California, U.S.A.).

The electrophoresis buffer used contained components in the following final concentrations:

25 mM	Tris base
192 mM	glycine
0.1% (w/v)	SDS

pH 8.3

Samples were diluted with an equal volume of 2X sample buffer:

250 mM	Tris/HCl, pH6.8
18.75% (v/v)	glycerol
4% (w/v)	SDS
100 mM	DTT
0.1% (w/v)	PAGE blue 83

The samples were boiled for 3 min and then loaded into sample wells.

Apparent molecular weights were determined with known standard proteins. For gels containing enzyme extracts a low molecular weight calibration kit was employed containing phosphorylase b (M.W. 94,000), bovine serum albumin (M.W. 67,000), ovalbumin (M.W. 43,000), carbonic anhydrase (M.W. 30,000), soybean trypsin inhibitor (M.W. 20,100) and α-lactalbumin (M.W. 14,400). For gels of *in vitro*-synthesised protein, 3µl of $[^{14}C]$methylated protein mixture containing myosin (M.W. 200,000), phosphorylase b (M.W. 94,000), bovine serum albumin (M.W. 67,000), ovalbumin (M.W. 43,000), carbonic anhydrase (M.W. 30,000) and lysozyme (M.W. 14,300) were used.

Electrophoresis was carried out for 15 hr at 12 mA constant current or for 5 hr at 35 mA constant current with cooling.

B. Staining of proteins

Proteins were visualised by staining of SDS/polyacrylamide gels for at least 60 min in the following solution:

30% (v/v)	methanol
7% (v/v)	acetic acid
0.25% (w/v)	PAGE blue 83

Excess stain was removed from the gel by washing in destain solution:

30% (v/v)	methanol
7% (v/v)	acetic acid

A piece of sponge foam was included in the destain solution to increase the efficiency of destaining.

C. Fluorography

If the purpose of the experiment was to analyse *in vitro*-synthesised protein, the proteins were fixed by soaking the gel in fixer solution:

30% (v/v)	methanol
10% (v/v)	acetic acid

The gel was then impregnated with En^{3}hance for 60 min, rinsed with distilled water for 50 min, dried and exposed to X-ray film (Fuji NIF-RX) at -70°C.

8. PRODUCTION OF ANTISERA

A. Preparation of protein for injection into rabbits

Proteins were electroeluted from polyacrylamide gel slices as described in Section II.6.D. Lyophilised protein samples were resuspended in phosphate-buffered saline to a final concentration of 250 μg/ml. Phosphate-buffered saline solution contained components in the following final concentrations:

8.1 mM	Na_2HPO_4
1.5 mM	KH_2PO_4
2.7 mM	KCl
137 mM	NaCl

pH 7.3

B. Immunisation protocol

Female New Zealand white rabbits were immunised using a method modified from that described by Mayer and Walker (1978). A control sample of 10 ml blood was removed from the ear veins prior to commencement of the immunisation protocol and also at 10 days following boost immunisations. The blood was allowed to clot at room temperature and then clarified by centrifugation at 4,000 x g for 15 min (4°C). The supernatant was removed and tested for the presence of antibodies to wheat endosperm ADP-glucose pyrophosphorylase as described in Section II.9.A.

The time course for immunisation is shown in Figure II.1. A total of 50 μg protein emulsified in Freund's complete adjuvant was injected subcutaneously at four sites on the shoulders and rump of each rabbit. After 21 days a boost immunisation of 25 μg protein emulsified in Freund's incomplete adjuvant was administered. This booster was repeated 21 days later. The absence of detectable antibodies at this stage suggested that a more rigorous immunisation protocol was required.

Rabbits were sensitised by injection with 1 ml Freund's complete adjuvant (no added protein) 3 days after the third blood sample was collected. Four days later 100 μg protein emulsified in Freund's complete adjuvant was administered. Two subsequent boost immunisations of 50 μg protein in complete adjuvant were then administered at 14 day intervals. Antiserum was collected from ear veins 10 days after the final immunisation, treated as described above and stored at -70°C in aliquots of 1 ml.

9. TITRATION OF ANTISERA

A. Solid phase ELISA

Determination of serum titre by solid phase ELISA was performed essentially as described by Niewola *et al* (1985). Wheat endosperm ADP-glucose pyrophosphorylase purified to the DEAE-Sepharose stage (Section II.6.B) was resuspended to a final protein concentration of 10 μg/ml, in 50 mM sodium bicarbonate buffer pH 9.6 containing 3 mM NaN_3. Aliquots (100 μl) of this solution were pipetted into microtitre wells (Nunc immunoplate, Gibco, Risley, Derbyshire) and

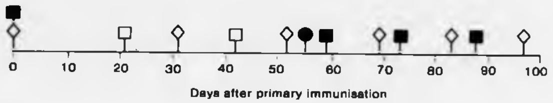


Figure II.1 : Time course for immunisation of rabbits with wheat endosperm ADP-glucose pyrophosphorylase. Rabbits were injected with protein in complete Freund's adjuvant (■), protein in incomplete Freund's adjuvant (□), or complete Freund's adjuvant only (●) at the times indicated. Blood was removed from the ear veins (◇) ten days after each boost immunisation. Time is shown in days following the primary immunisation of rabbits

incubated overnight at 4°C to allow the protein to adsorb. The plates were then washed at room temperature three times with phosphate-buffered saline containing 0.5% (v/v) Tween 20. Washes were of 5 minutes duration. Following this, 100 µl aliquots of phosphate-buffered saline containing 8% (w/v) bovine serum albumin were pipetted into the wells and plates incubated at 4°C for 2 hr. After washing the plates as before, serial dilutions of rabbit serum in a final volume of 100 µl, were added to the wells. The dilutions were prepared in triplicate and plates were subsequently incubated at 4°C for 4 hr. The plates were again washed and 100 µl aliquots of a 1/4000 dilution of horseradish peroxidase-labelled goat anti- rabbit IgG serum were added to the wells. Plates were incubated at 4°C for 2 hr and then washed as before. The amount of peroxidase-labelled IgG bound to anti- wheat endosperm ADP-glucose pyrophosphorylase IgG was quantified by the addition of 100 µl of freshly prepared substrate solution:

50 mM	Na_2HPO_4
25 mM	citric acid
0.08% (w/v)	*o*-phenylenediamine
0.02% (w/v)	urea hydrogen peroxide

After 5-10 min incubation at room temperature in the dark, the reactions were stopped by addition of 50 µl of 500 mM citric acid. The microtitre plate was inserted into the sample chamber of a Multiskan Spectrophotometer (Flow Laboratories, Rickmansworth, Hertfordshire) and the absorbance of samples at 450 nm was determined.

10. IMMUNOPRECIPITATION OF PROTEIN

A. Immunoprecipitation of enzyme activity

To confirm the specificity of the anti- wheat endosperm ADP-glucose pyrophosphorylase serum, the ability of it to precipitate enzyme activity *in vitro* was tested. Endosperms (1 g) aged 21 d.p.a. were homogenised in 10 ml of 50 mM potassium phosphate buffer pH 7.5 containing 0.1 mM glutathione. The homogenate was centrifuged at 39,000 x g for 20 min (4°C) and the supernatant retained. Immunoprecipitation samples were prepared in triplicate and contained components in the following final concentrations:

30% (v/v)	enzyme extract
10% (v/v)	antiserum

The final volume of the immunoprecipitation reaction was 300 µl. Control samples contained phosphate-buffered saline or preimmune serum in place of antiserum. Following incubation at 4°C for 24 hr, 20 mg of protein A-Sepharose, equilibrated in enzyme extraction buffer, was added to each sample. After a second incubation at 4°C for 16 hr with continuous mixing, the protein A-Sepharose was removed by centrifugation at 12,000 x g for 5 min.

The supernatants were assayed for activity of ADP-glucose pyrophosphorylase as described in Section II.3.A.1. To test for non-specific immunoprecipitation of enzyme activity, the activities of two marker enzymes, UDP-glucose pyrophosphorylase and malate dehydrogenase were assayed as described in Sections

II.3.B and II.3.C respectively.

The protein A-Sepharose pellets were washed three times with 1 ml of enzyme extraction buffer. Washes were carried out for 60 min at 4°C, with continuous mixing. The washed pellets were then added directly to an assay mixture for measurement of ADP-glucose pyrophosphorylase activity as described in Section II.3.A.1. Assays were carried out in this case with continuous mixing to keep the Sepharose beads in suspension. Assays for marker enzymes in the pellet fractions were not possible because the Sepharose beads interfered with spectrophotometric determinations.

B. Immunoprecipitation of *in vitro*-synthesised protein

Poly(A)-containing RNA samples were translated as described in Section II.13.A. Immunoprecipitation of *in vitro*-translation products was carried out using a modification of the method described by Speirs and Brady (1981). Reactions were carried out in sterile Eppendorf microcentrifuge tubes. The immunoprecipitation buffer contained components in the following final concentrations:

1 M	Tris/HCl, pH 8.6
5% (v/v)	Triton X-100
0.9% (w/v)	SDS
4.3 μg/ml	PMSF
4.3 mM	DTT

To 3.3 μl of immunoprecipitation buffer was added 10 μl of *in*

vitro-translated protein mixture (3-8 x 10^4 cpm/μl) and 4 μl of anti- wheat endosperm ADP-glucose pyrophosphorylase serum (or 1 μl of anti- spinach leaf ADP-glucose pyrophosphorylase serum). Control samples contained 4 μl of preimmune serum in place of the antiserum, or 4 μl of purified wheat endosperm ADP-glucose pyrophosphorylase (4 μg/μl) in addition to the standard reaction components. Samples were incubated at 4°C for 24 hr and then 5 mg protein A-Sepharose were added. Following a further incubation at 4°C for 4 hr with continuous mixing, the samples were washed extensively in phosphate-buffered saline containing 0.5% (v/v) Tween 20. Finally, the protein A-Sepharose-IgG-protein pellets were resuspended in 100 μl of 2X electrophoresis sample buffer (Section II.7.A), boiled for 5 min and quickly centrifuged to pellet the protein A sepharose. Samples were analysed on SDS/polyacrylamide gels as described in Section II.7.

11. WESTERN BLOT IMMUNOELECTROPHORESIS

A. Transfer of proteins onto nylon membranes

Following gel electrophoresis of proteins as described in Section II.7.A, proteins were transferred onto nylon membranes (Hybond N) as described by Towbin *et al* (1979). The unstained gel and nylon membrane were soaked for 30 min in Towbin's buffer:

25 mM	Tris base
192 mM	glycine
20% (v/v)	methanol

pH 8.3

The gel and nylon membrane were sandwiched together taking care to remove air bubbles. The sandwich was placed into a Bio-Rad Trans-blot Cell containing Towbin's buffer. A constant current of 250 mA was applied for 16 hr with cooling . The nylon membrane was washed briefly in the same buffer and allowed to dry at room temperature.

B. Probing protein blots with antisera

Protein blots were probed with antisera according to the instructions of the supplier of Hybond N membranes. The membrane was incubated at 45°C for 20 hr, in 200 ml of phosphate-buffered saline containing 8% (w/v) bovine serum albumin. Following this, the membrane was incubated for 1 hr at room temperature in a sealed plastic bag containing a solution of 2% (w/v) bovine serum albumin, 0.4% (v/v) antiserum in phosphate-buffered saline. The membrane was washed three times in phosphate-buffered saline containing 0.5% (v/v) Tween 20. Finally, the membrane was incubated at room temperature for 1 hr in phosphate-buffered saline containing 2% (w/v) bovine serum albumin and 0.3 μCi/ml ^{125}I-protein A. Following washing as before, the membrane was exposed to a pre-flashed X-ray film (Fuji NIF-RX) with an intensifying screen (Du Pont, Cronex Lightening Plus) at -70°C.

12. EXTRACTION AND PURIFICATION OF RNA

A. Extraction of polysomal RNA from wheat

Polysomes were prepared from wheat endosperms aged 15 d.p.a., or from 7-day old expanding first wheat leaves, according to Greene (1981). All buffers were autoclaved and sterile disposable plastic ware was used where possible to minimise contamination by exogenous ribonuclease. All reagents used for RNA extraction and purification were kept separate from other laboratory stocks and were dispensed without the aid of spatulas. Corex centrifuge tubes and other glassware were baked overnight at 220°C to destroy RNase activity. Stoneware mortars and pestles were washed with 50% (v/v) nitric acid and then rinsed with sterile double-distilled water before use. Disposable gloves were worn at all stages of the purification.

All steps were carried out at 4°C. Approximately 10 g of plant material were dissected into liquid nitrogen and stored until required. The tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle and transferred to a clean chilled mortar containing 50 ml of modified Larkin's buffer:

200 mM	Tris/HCl, pH 8.5
200 mM	sucrose
25 mM	$MgCl_2$
60 mM	KCl
5 mM	DTT
2 mM	AMP
0.1% (w/v)	heparin

Following further homogenisation of the tissue in buffer, starch and cell debris were removed by centrifugation at 16,000 x g for 30 min. The supernatant was used as a source of free cytoplasmic polysomes. Membrane-bound polysomes were released by resuspension of the pellet in a further 50 ml of modified Larkin's buffer containing 2% (v/v) Triton X-100. Following an additional centrifugation as before, the supernatant was retained as a source of membrane-bound polysomes.

The supernatants were centrifuged at 250,000 x g for 150 min through a 700 mM sucrose cushion overlaying a 1.4 M sucrose cushion (5 ml and 4 ml volumes respectively) to pellet the polysomes. Sucrose cushions were prepared by appropriate dilution of 2.1 M sucrose in modified Larkin's buffer.

For the extraction of RNA the polysome pellets were resuspended in 5 ml of RNA extraction buffer:

50 mM	Tris/HCl, pH 9.0
100 mM	NaCl
10 mM	EDTA
0.5% (w/v)	SDS

The suspension was extracted twice with an equal volume of phenol:chloroform [1:1 (v/v)]. After each extraction the samples were centrifuged at 12,000 x g for 10 min and the aqueous phase recovered. RNA was precipitated from the aqueous phase by addition of 0.05 volumes of 3 M sodium acetate pH 6.0 and 2.5 volumes of ice-cold ethanol. Following an incubation at -20°C overnight, the RNA was recovered by centrifugation at 20,000 x g for 20 min, washed twice in ice-cold 80% (v/v) ethanol, dried under vacuum and resuspended in sterile double-distilled water. Polysomal RNA samples were then stored at -70°C.

B. Purification of poly(A)-containing RNA

Poly(A)-containing RNA was purified from total polysomal RNA on oligo(dT)-cellulose columns according to Aviv and Leder (1972). All steps were carried out at room temperature unless otherwise specified. A sterile silanised glass column (1 cm x 5 cm) was filled with 0.5 g oligo(dT) cellulose. The resin was washed with 20 column volumes (w/v) of RNA binding buffer:

10 mM	Tris/HCl, pH 7.2
400 mM	NaCl
0.3% (w/v)	SDS

The column was then sterilised by washing with 5 ml of 100 mM NaOH followed by incubation for 30 min in the same solution. Excess NaOH was removed by washing the column with sterile double-distilled water. The column was then equilibrated in RNA binding buffer.

The RNA sample was adjusted to a concentration of 5 mg/ml in a final volume of 500 µl of RNA binding buffer. This solution was then applied to the column at a flow rate of 50 µl/min. The flow rate was maintained at this level for the remainder of the procedure. The column eluant was collected and reapplied to the column three times. Non-poly(A)-containing RNA was eluted from the column by washing it with 8 ml of RNA binding buffer. Fractions of

1ml were collected and the ultraviolet absorbance at 260 nm was measured. Poly(A)-containing RNA was eluted from the column by washing with 4 ml of elution buffer:

10 mM	Tris/HCl, pH 7.2
0.3% (w/v)	SDS

Fractions of 500 μl volume were collected and the ultraviolet absorbance at 260 nm was measured. Fractions with absorbance greater than 0.04 at 260 nm were pooled; poly(A)-containing RNA was precipitated by addition of 0.05 volumes of 3 M sodium acetate pH 6.0 and 2.5 volumes of ice-cold ethanol. Following an overnight incubation at -20°C, the RNA was recovered by centrifugation at 20,000 x g (4°C) for 30 min. The RNA pellet was washed twice with ice-cold 80% (v/v) ethanol, dried under vacuum and resuspended in 20 μl of sterile double-distilled water. Samples were stored at -70°C. Generally one cycle of purification on oligo(dT)-cellulose was sufficient to provide poly(A)-containing RNA suitable for *in-vitro* translation and Northern blot experiments. For cDNA synthesis experiments, the wheat endosperm polysomal RNA was purified by two cycles on oligo(dT) cellulose.

C. Estimation of RNA quality and quantity

The amount of RNA present in samples was calculated by measuring the ultraviolet absorbance at 260 nm in a Pye Unicam Model SP8-400 spectrophotometer. An RNA solution with a concentration of 40 μg/ml was taken as having an absorbance of 1.0

in a 1.0 cm path length (Maniatis *et al*, 1982).

The quality of RNA samples was assessed by the ability of RNA to direct the cell-free synthesis of protein as described in Section II.13.A.

13. *IN VITRO* PROTEIN SYNTHESIS

A. Cell-free translation of RNA samples

RNA samples were translated *in vitro* in the presence of L-[^{35}S]methionine using a mRNA-dependent, amino acid-depleted rabbit reticulocyte lysate according to the supplier's instructions. *In vitro* synthesis reactions were carried out in sterile Eppendorf microcentrifuge tubes and contained components in the following final concentrations in a volume of 25 μl:

70% (v/v)	reticulocyte lysate
770 μM	magnesium acetate
116 μM	potassium acetate
50 μM	amino acids less methionine
1 mCi/ml	L-[^{35}S]methionine (>800 Ci/mmol)
40 μg/ml	poly(A)-containing RNA (or 400 μg/ml total polysomal RNA)

The RNA was the last component added to the reaction mixtures. Samples were incubated at 30°C for 1 hr.

B. Measurement of L-[^{35}S]methionine incorporated into protein

At the end of the translation incubation period, 1 µl aliquots of the translation mixture were removed and added to 500 µl of 1 M NaOH containing 5% (v/v) H_2O_2 (100 volumes). This mixture was incubated at 37°C for 10 min to hydrolyse amino acyl-tRNA complexes. Following this incubation, protein was precipitated by addition of 2 ml ice-cold 25% (w/v) TCA, 2% (w/v) casein hydrolysate and incubation on ice for 1 hr. The protein was collected by filtration of samples through 0.45 µm filter discs (Millipore SA, 67120 Molsheim, France). The filters were washed three times with 5 ml ice-cold 10% (w/v) TCA and then dried at 65°C for 10 min. Filters were subsequently counted in 5 ml scintillant (Beckman MP) in an LKB Rackbeta scintillation counter.

14. cDNA LIBRARY CONSTRUCTION

A. First-strand cDNA synthesis

cDNA was synthesised using reagents from the Amersham cDNA synthesis system according to the manufacturer's instructions. This method is a modification of that described by Gubler and Hoffman (1983). Unfortunately, the manufacturer of the cDNA synthesis system has not published the composition of buffers used for the first- and second-strand cDNA syntheses, or the concentrations of sodium pyrophosphate, deoxynucleotide triphosphates and olig(dT)$_{12-18}$ primer solutions used in the first-strand synthesis reaction. Therefore the composition of reaction mixtures shown below does not provide detailed final concentrations of reagents.

The first-strand cDNA was synthesised from wheat endosperm poly(A)-containing RNA which had been purified by two cycles on oligo(dT)-cellulose as described in Section II.12.B. The reaction mixture for first-strand cDNA synthesis contained the following final concentrations of components:

1X	first-strand synthesis buffer
1X	sodium pyrophosphate solution
1.25 units/μl	human placental RNase inhibitor
1X	dATP, dCTP, dGTP, dTTP mixture
1X	oligo(dT)$_{12-18}$ primer
250 μCi/ml	[α-^{32}P]dCTP (> 400 Ci/mmol)
80 μg/ml	poly(A)-containing RNA
2.0 units/μl	AMV reverse transcriptase

Reactants were added in the order given. The final volume of the reaction mixture was 30 μl. The reaction was incubated at 42^{0}C for 2 min prior to the addition of reverse transcriptase. Following addition of the enzyme the mixture was incubated for a further 1 hr at 42^{0}C and then placed on ice.

For the estimation of cDNA yield from the first-strand synthesis reaction, a 1 μl aliquot of the reaction mixture was diluted into 20 μl of sterile distilled water and 2 μl of the dilution was spotted onto a disc of DE81 filter paper (Whatman). The total radioactivity was determined by counting the filter in an LKB Rackbeta scintillation counter. The radioactivity incorporated into cDNA was determined by washing the filter six times with 500 mM Na_2HPO_4, twice with double-distilled water and twice with ethanol. The filter was dried and counted as before. The yield of first-strand cDNA was calculated using the following relation, which assumes that the average molecular weight of a nucleotide base in DNA is 350:

$$\text{Yield (ng)} = 350 \times 4 \times [\text{dCTP}]\ (\text{nmol}) \times \%\ {}^{32}\text{P incorporated}$$

B. Second-strand cDNA synthesis

The second-strand cDNA synthesis reaction mixture contained the following final concentrations of components in a final volume of 150 µl:

20% (v/v)	first-strand reaction mixture
1X	second-strand reaction buffer
500 µCi/ml	[α-^{32}P]dCTP (> 400 Ci/mmol)
0.016 units/µl	*E.coli* RNase H
0.46 units/µl	*E.coli* DNA polymerase I

The reactants were added in the order listed. The reaction mixture was mixed gently and incubated sequentially at 12°C for 1 hr and at 22°C for 1 hr. The reaction was terminated by heating to 70°C for 10 min and then the tube was placed on ice.

For the estimation of cDNA yield from the second-strand synthesis reaction, a 1 µl aliquot of the reaction mixture was removed and treated as described in Section II.14.A. Allowing for the amount of radioactivity present in the first-strand reaction mixture, the following relation was used to calculate the percentage incorporation of [α-^{32}P]dCTP during the second strand synthesis reaction:

$$\text{Incorp.} = \frac{[\text{c.p.m. incorp.(total)} - \text{c.p.m. incorp.(1st strand)}/5]\ \text{x100}}{[\text{input c.p.m.(total)} - \text{input c.p.m.(1st strand)}/5]}$$

The yield of cDNA was then calculated using the relation shown in Section II.14.A.

C. Creation of blunt-ended cDNA molecules

Double-stranded cDNA molecules were blunt-ended in a reaction mixture containing components in the following final concentrations:

97.0% (v/v)	second-strand reaction mixture
1 mM	Tris/HCl, pH 7.5
10 mM	NaCl
0.7 mM	$MgCl_2$
0.7 mM	2-mercaptoethanol
5 mM	dATP, dCTP, dGTP, dTTP (each)
1.2 units/µl	T_4 DNA polymerase

The reaction mixture was incubated at 37°C for 30 min, then the reaction was stopped by addition of 15 µl of 250 mM EDTA pH 8.0. The sample was extracted once with an equal volume of phenol: chloroform [1:1 (v/v)]. Following centrifugation at 12,000 x g for 5 min, the aqueous phase was chromatographed on a 2.2 ml column of Sephadex G-50 fine, packed into a Pasteur pipette. The column was previously equilibrated with 0.1X *Eco*RI methylase buffer:

8 mM	Tris/HCl, pH 8.0
0.1 mM	EDTA

The radioactivity corresponding to the first radioactive peak eluting from the column was pooled. The cDNA was subsequently concentrated by lyophilisation to 1/10th of the original volume.

D. Methylation of cDNA

Blunt-ended cDNA molecules were methylated using a method modified from that of Huynh *et al* (1985). The reaction mixture contained components in the following final concentrations:

ca. 16 µg/ml	cDNA molecules
80 mM	Tris/HCl, pH 8.0
1 mM	EDTA
200 µg/ml	bovine serum albumin
80 µM	s-adenosyl methionine
0.8 units/µl	*Eco*RI methylase

The volume of the reaction mixture was 50 µl. The reaction was incubated at 37°C for 1 hr and then extracted with phenol:chloroform [1:1 (v/v)] followed by chloroform.

E. Size fractionation of cDNAs

Methylated cDNAs were size-fractionated on a column (0.7 cm x 8.5 cm) of Bio-gel A150m (100-200 mesh) according to Huynh *et al* (1985). The Biogel was washed three times in column buffer before packing into the column. The column buffer was as follows:

10 mM	Tris/HCl, pH 7.5
100 mM	NaCl
1 mM	EDTA

The packed column was washed with 5 column volumes of buffer (v/v) and the cDNA sample gently loaded onto the top of the resin. The volume of the sample was 50 μl. The flow rate of the column was maintained at 25 μl/min and fractions of 50 μl were collected.

Aliquots (2 μl) were removed from each fraction containing radioactivity and these were analysed on a 1.2% (w/v) agarose gel (see Section II.18.A). The gel was dried under vacuum and exposed to X-ray film (Fuji NIF-RX). Those fractions containing cDNAs > 500 basepairs in size were pooled and the DNA precipitated by addition of 0.1 volumes of 3 M sodium acetate, pH 6.0, and 2.0 volumes ice-cold ethanol. Following an overnight incubation at -20°C the cDNA was recovered by centrifugation at 12,000 x g for 10 min. The DNA pellet was washed once with ice-cold 70% (v/v) ethanol, air-dried and resuspended in 5 μl of sterile TE buffer:

10 mM	Tris/HCl, pH 8.0
1 mM	EDTA

F. Addition of synthetic *Eco*RI linkers to cDNAs

1.Phosphorylation of linkers

Synthetic *Eco*RI linker molecules were phosphorylated and ligated to cDNAs essentially according to Huynh *et al* (1985). Firstly, linkers were labelled at their 5'-termini by phosphorylation with T_4 polynucleotide kinase. The reaction mixture contained components in the following final concentrations in a volume of 25 μl:

200 μg/ml	dephosphorylated *Eco*RI linkers
50 mM	Tris/HCl, pH 7.5
10 mM	$MgCl_2$
10 mM	DTT
800 μCi/ml	[γ-^{32}P]ATP (3 mCi/mmol)
1.2 units/μl	T_4 polynucleotide kinase

Following incubation at 37°C for 15 min, the reaction was chased by addition of 25 μl of the following mixture:

50 mM	Tris/HCl, pH 7.5
10 mM	$MgCl_2$
10 mM	DTT
1 mM	ATP, pH 7.6
1.2 units/μl	T_4 polynucleotide kinase

The reaction mixture was incubated for 45 min at 37°C and linkers were stored at -20°C until required.

2. Linker-cDNA ligation

Size-fractionated cDNAs (> 500bp) were ligated to 5'-end-labelled *Eco*RI linkers using T_4 DNA ligase. The size-fractionated cDNAs were diluted with an equal volume of the following mixture:

100 mM	Tris/HCl, pH 7.5
20 mM	$MgCl_2$
20 mM	DTT
2 mM	ATP
10 μg/ml	^{32}P-labelled *Eco*RI linkers
0.63 units/μl	T_4 DNA ligase
0.86 units/μl	T_4 RNA ligase

The final volume of the ligation reaction was 10 μl. The reaction was incubated at 12°C for 20 hr and then terminated by heating at 65°C for 10 min. Excess linkers were cleaved from the linkered cDNAs by restriction endonuclease digestion with *Eco*RI enzyme as described in Section II.19. The solution was then extracted with phenol:chloroform [1:1 (v/v)], centrifuged at 12,000 x g for 5 min and the aqueous phase chromatographed on a column (0.7 cm x 8.5 cm) of Sepharose CL-4B. Conditions were the same as for size-fractionation of cDNA molecules (see Section II.14.E). Fractions corresponding to the first peak of radioactivity were pooled and DNA was precipitated by addition of 0.1 volumes of 3 M sodium acetate, pH 6.0, and 2.0 volumes of ice-cold ethanol. Following an overnight incubation at -20°C, the linkered DNA was recovered by centrifugation at 12,000 x g for 10 min. the DNA pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in 3 µl sterile distilled water.

G. Ligation of cDNAs to λgt11 vector DNA

Commercial bacteriophage λgt11 arms were supplied pre-digested with *Eco*RI and phosphatased. Following test ligations and *in-vitro* packaging to check that the bacteriophage arms were efficiently phosphatased, the linkered cDNAs were ligated to λgt11 arms. The linkered cDNAs were diluted with an equal volume of the following mixture:

100 mM	Tris/HCl, pH 7.5
20 mM	$MgCl_2$
20 mM	DTT
2 mM	ATP
400 µg/ml	phosphatased, *Eco*RI digested λgt11
1.26 units/µl	T_4 DNA ligase

Ligation reactions were carried out in a final volume of 6 µl and incubated at 12°C for 20 hr. Recombinant λgt11 molecules were packaged *in vitro* as described in Section II.14.H.

H. *In vitro* packaging of recombinant λgt11 molecules

Recombinant λgt11 molecules were packaged using the *in vitro* packaging extract Gigapack Gold as recommended by the supplier.

Approximately 500 ng of concatamerised DNA was added to the supplied Freeze/Thaw Extract and to this mixture was added 15 µl of the supplied Sonic Extract. The contents of the tube were mixed very gently taking care not to introduce air bubbles. The packaging reactions were incubated at 22°C for 2 hr. To the packaged DNA was added 500 µl of phage buffer:

50 mM	Tris/HCl, pH 7.5
100 mM	NaCl
10 mM	$MgSO_4$
0.01% (w/v)	gelatin

Bacteriophage were stored over chloroform at 4°C in sealed ampoules. The percentage of recombinant bacteriophage was determined by plating a small aliquot onto *E.coli* strain Y1088 as described in Section II.15.B and incubating plates at 42°C overnight.

15. PROPAGATION OF BACTERIOPHAGE λgt11

A. Preparation of plating bacteria

Plating bacteria were prepared according to Maniatis *et al* (1982). A colony of *E.coli* strain Y1088 or *E.coli* strain Y1090 was inoculated into 20 ml of L-broth containing 0.2% (w/v) maltose and 50 µg/ml ampicillin. L-broth contained the following final concentrations of reagents:

1% (w/v)	bactotryptone
0.5% (w/v)	yeast extract
0.5% (w/v)	NaCl
1% (w/v)	α-D-glucose

pH 7.0

The culture was incubated at 37°C overnight. Cells were harvested by centrifugation at 1,500 x g for 15 min and the pellet was gently resuspended in 8 ml of ice-cold 10 mM $MgSO_4$. The concentration of plating cells was adjusted to $2x10^9$ cells/ml. Plating cells were stored at 4°C for up to one week.

B. Plating bacteriophage λgt11

Bacteriophage were propagated on bacterial lawns by incubating 200 μl of plating bacteria with $< 1x10^4$ p.f.u. of λgt11 for 15 min at room temperature. If the purpose of the experiment was to titre bacteriophage, a small number of phage (ca. 100 p.f.u.) were used for the infection of cells. If the purpose of the experiment was to prepare confluently lysed plates, the amounts of plating bacteria and bacteriophage used in the infection were varied to 700 μl cells and $1x10^6$ - $1x10^8$ p.f.u. respectively. The *E.coli* strains used are described elsewhere in the text in sections corresponding to particular experiments.

Warm BBL top agar (3 ml), containing 0.2% (w/v) maltose and 50 μg/ml ampicillin, was added to the infected cells. BBL top agar contained reagents in the following final concentrations:

1% (w/v)	trypticase
0.5% (w/v)	NaCl
0.65% (w/v)	bactoagar

pH 7.0

This mixture was poured quickly onto dry 85 mm L-broth plates containing 50 μg/ml ampicillin. L-broth plates were made by supplementing L-broth (see Section II.15.A) with 1.5% (w/v) bactoagar. If the purpose of the experiment was to determine the percentage of recombinant bacteriophage in a cDNA library the plates were also supplemented with 20 μg/ml IPTG and 80 μg/ml X-gal. If the purpose of the experiment was to produce plate lysate stocks of bacteriophage, the plates were incubated at 37°C overnight. The confluently lysed plates were then washed with 5 ml phage buffer at 4°C overnight. The eluted phage were recovered with the phage buffer and stored at 4°C over chloroform.

C. Propagation of bacteriophage in liquid culture

Bacteriophage λgt11 was propagated in liquid culture using a method modified from that of Maniatis *et al* (1982). To produce 200 ml of liquid lysate culture, 700 μl of *E.coli* strain Y1088 plating cells were infected with ca. 1x10^{8} p.f.u. of λgt11 at room temperature for 15 min. Generally, to obtain bacteriophage at sufficiently high titre for this infection, prior preparation of plate lysate stocks as described in Section II.15.B was necessary.

The infected cells were transferred to 200 ml of prewarmed (37°C) YT medium, containing 0.2% (w/v) maltose, 10 mM $MgCl_2$ and

50 µg/ml ampicillin. YT medium contained reagents in the following final concentrations:

0.8% (w/v)	bactotryptone
0.5% (w/v)	yeast extract
0.25% (w/v)	NaCl

Cultures were incubated at 37°C overnight with shaking (ca. 150 r.p.m.). At this stage cell lysis was usually apparent. Chloroform was added to the cultures to a final concentration of 2.5% (v/v) and the cultures were incubated for an additional 30 min at 37°C to lyse any remaining infected cells. Bacteriophage were subsequently harvested as described in Section II.17.A.

16. SCREENING OF cDNA LIBRARIES

A. Screening cDNA libraries with antibody probes

A wheat leaf cDNA library obtained from Dr C. Raines and Dr T.A. Dyer (Plant Breeding Institute,Trumpington, Cambridge) was screened with antibody probes using a modification of the method described by Young and Davis (1983b). Antibodies were pre-treated to remove components which recognise *E.coli* antigens and the screening was carried out as described below.

1. Pre-treatment of antibodies

An *E.coli* strain Y1088 plate lysate was prepared as described in Section II.15.B, using non-recombinant λgt11 to infect cells. The plate lysate was clarified by centrifugation at 4,000 x g for 10 min and then placed in a sealed plastic bag with a 200 cm^2 sheet of nitrocellulose membrane. Following incubation at 37°C for 2 hr the plate lysate was removed and the filter incubated for an additional 1 hr at 37°C with 50 ml of blocking solution:

50 mM	Tris/HCl, pH 7.5
150 mM	NaCl
2% (w/v)	bovine serum albumin
0.02% (w/v)	NaN_3

Anti-wheat endosperm ADP-glucose pyrophosphorylase serum was diluted 1/100 in fresh blocking solution and anti-spinach leaf ADP-glucose pyrophosphorylase serum was diluted 1/250 in fresh blocking solution. The nitrocellulose filter was subsequently incubated in diluted antibody solution for 24 hr at 37°C. The pre-treated antibody solution was used immediately but could be reused over a three week period if stored at 4°C between experiments.

2. Immunological screening

Bacteriophage ($1X10^4$ p.f.u) were plated onto *E.coli* strain Y1090 as described in Section II.15.B. Plates were incubated at 42°C for 3 hr. Following this incubation, nitrocellulose membrane discs (82 mm diameter) which had been been soaked in 10 mM IPTG and dried were carefully overlaid the plates. The plates were transferred to a 37°C incubator for a further 4 hours. The plates were subsequently removed to room temperature and the positions of the overlaid filters were marked by stabbing through the filter into the agar with a sterile needle. The filters were carefully peeled off the plates and washed at room temperature for 15 min in 1XTBS containing 0.02% (w/v) NaN_3. The 1XTBS solution contained the following final concentrations of reagents:

50 mM	Tris/HCl, pH 7.5
150 mM	NaCl

Washes were carried out in a plastic sandwich box using at least 250 ml of 1XTBS solution for 10 filters. The filters were subsequently incubated with blocking solution (Section II.16.A.1) in sealed plastic bags, for 1 hr at 37°C.

The blocked filters were incubated with pre-treated antibody (Section II.16.A.1), allowing 2-3 ml of antibody solution per filter disc. Incubations were carried out overnight at 37°C in sealed plastic bags. Following this incubation, the filters were washed three times at room temperature, using 150 ml of 1XTBS per wash. Washes were of 30 min duration.

For detection of bound IgG, filters were incubated with 25 ml of 1XTBS solution containing 0.1% (v/v) horseradish peroxidase-labelled goat-anti-rabbit IgG serum and 2% (w/v) bovine serum albumin. Incubations were carried out in sealed plastic bags for 90 min at 37°C. The filters were then washed as before, rinsed twice in sterile distilled water and placed in 100 ml of substrate solution:

10 mM	Tris/HCl, pH 7.5
0.05% (w/v)	4-chloro-1-napthol
0.05% (v/v)	H_2O_2 (100 volumes)

When the colour had developed sufficiently, the filters were washed under running water to stop the reaction.

The positions of positive signals on the filters were keyed back to the original plates. Plaques identified as expressing wheat leaf ADP–glucose pyrophosphorylase epitopes were removed from the plates with a sterile Pasteur pipette. Bacteriophage were eluted into 500 µl phage buffer to obtain stocks for the next round of screening.

The procedure was repeated, using the following approximate numbers of p.f.u. per plate for each subsequent round of screening:

Screening round	p.f.u./plate
2	1,000
3	500
4+	100

In this way positive signals were purified until every plaque on the plate produced a positive signal. The plaque-purified bacteriophage stocks were stored at 4°C over chloroform.

B. Screening cDNA libraries with DNA probes

The wheat endosperm cDNA library constructed as described in Section II.14 was screened without prior amplification for sequences encoding ADP-glucose pyrophosphorylase. The method used was modified from that described by Maniatis *et al* (1982). The DNA probe used for the screening procedure was purified from a wheat leaf cDNA clone (designated WL:AGA.1), which had been isolated using anti-spinach leaf ADP-glucose pyrophosphorylase serum as described in Section II.16.A.

Approximately 1x10^3 p.f.u. bacteriophage were plated onto *E.coli* strain Y1088 as described in Section II.15.B. Plates were incubated at 37°C overnight and then placed at 4°C for 15 min to harden the BBL top agar overlay. Nitrocellulose filter discs (82mm diameter) were then carefully overlaid at room temperature. After 2 min the positions of the filters on the plates were marked by stabbing through the filters into the agar with a sterile needle. The filters were carefully peeled off the plates and placed bacteriophage side-up onto 3MM filter paper (Whatman) soaked in denaturing solution:

500 mM	NaOH
1.5 M	NaCl

After 5 min the filters were transferred in the same orientation onto 3MM filter paper soaked in neutralising solution:

500 mM	Tris/HCl, pH 7.5
1.5 M	NaCl

After an additional 5 min incubation the filters were transferred in the same orientation onto 3MM filter paper soaked in 2XSSC solution. A stock solution of 20XSSC contained the following final concentrations of reagents:

300 mM	sodium citrate, pH 7.0
3 M	NaCl

After 5 min incubation in 2XSSC, the filters were dried at room temperature and then baked under vacuum at 80°C for 2 hr.

The filters were prehybridised by incubation for 4 hr at 65°C in 200 ml hybridisation buffer:

5X	SSPE solution *
0.1% (w/v)	bovine serum albumin
0.1% (w/v)	SDS
0.1% (w/v)	Ficoll (mol. wt 400,000)
0.1% (w/v)	polyvinyl pyrrolidone
0.01% (w/v)	denatured herring sperm DNA

* A stock solution of 20XSSPE contained the following final concentrations of reagents:

3.6 M	NaCl
200 mM	NaH_2PO_4, pH 6.8
20 mM	EDTA

Following prehybridisation, the filters were placed in sealed plastic bags (10 filters/ bag) containing ca. 2 ng/ml of the nick-translated cDNA insert of clone WL:AGA.1 in 30 ml of fresh hybridisation buffer. The DNA probe was nick-translated as described in Section II.22.A and denatured by boiling for 5 min before addition to the hybridisation buffer. After an overnight incubation at 65°C the filters were washed three times with 2XSSC containing 0.1%(w/v) SDS. Washes were carried out at 65°C for 30 min. The filters were then exposed to X-ray film (Fuji NIF-RX) with an intensifying screen at -70°C.

The positions of positive signals were keyed back to the original plates. Plaques identified as containing sequences homologous to the probe DNA were removed with a sterile Pasteur pipette. The bacteriophage were eluted into 500 μl of phage buffer to obtain stocks for the next round of screening.

The screening procedure was repeated using the following p.f.u. per plate for each subsequent round of screening:

Screening round	p.f.u./plate
2	500
3+	100

In this way positive signals were purified until every plaque produced a positive signal. The plaque-purified bacteriophage stocks were stored at 4°C over chloroform.

17. EXTRACTION, PURIFICATION AND ANALYSIS OF DNA

A. Preparation of bacteriophage DNA from liquid lysate cultures

Bacteriophage DNA was prepared from liquid lysates using a method modified from that of Maniatis *et al* (1982). Bacteriophage were firstly propagated in liquid culture as described in Section

II.15.C.

RNase (1 μg/ml) and DNase (1 μg/ml) were added to the chloroformed cultures. Following incubation at room temperature for 30 min the cultures were made to 1 M NaCl and placed on ice for 1 hr. Cell debris was removed by centrifugation at 10,000 x g for 15 min (4°C) and the supernatant made to 10% (w/v) PEG_{8000}. Following complete dissolution of the PEG_{8000} the bacteriophage were precipitated by incubation of cultures on ice for 1 hr. Bacteriophage were harvested by centrifugation at 10,000 x g for 15 min (4°C) and then resuspended gently in 5 ml phage buffer. Excess PEG_{8000} was removed by extraction of the bacteriophage suspension with an equal volume of chloroform. Following centrifugation at 4,000 x g for 10 min (4°C) the aqueous phase was treated with RNase and DNase as before.

Bacteriophage were lysed by addition of 0.5% (w/v) SDS and 50 μg/ml proteinase K and subsequent incubation at 65°C for 1 hr. The solution was deproteinised by extraction twice with phenol:chloroform [1:1 (v/v)] and once with chloroform. The solution was centrifuged at 4,000 x g for 10 min following each extraction and the aqueous phase was retained. DNA was precipitated from the aqueous phase by addition of 0.1 volumes of 3 M sodium acetate, pH 6.0, plus 2 volumes of ice-cold ethanol. Following overnight incubation at -20°C, the phage DNA was recovered by centrifugation at 12,000 x g for 15 min (4°C). The DNA pellet was dried under vacuum and resuspended in 200-500 μl of sterile double-distilled water.

DNA prepared from liquid lysate cultures was purified further by chromatography on Sepharose CL-4B columns (1.0 cm x 20 cm). The column buffer used was TE buffer and fractions of 1 ml were collected. Bacteriophage DNA generally eluted in fractions 4-6 inclusive. DNA was precipitated as before and finally resuspended in 200 µl TE buffer.

B. Preparation of Bacteriophage DNA from plate lysate cultures

For a large scale preparation of bacteriophage DNA, twenty plate lysates were prepared as described in Section II.15.B. Bacteriophage particles were subsequently precipitated from plate lysates by combined NaCl/ PEG_{6000} precipitation as described in Section II.17.A.

The bacteriophage pellet obtained by NaCl/PEG_{6000} precipitation was resuspended in 20 ml of phage buffer and extracted with an equal volume of chloroform. Following centrifugation at 4,000 x g for 10 min (4°C), the aqueous phase was removed and retained. Solid CsCl (0.75 g) was added per ml of solution. This was then transferred to 35 ml polyallomer centrifuge tubes (Sorvall, Du Pont) and centrifuged at 196,800 x g for 20 hr (4°C). The bacteriophage band was removed with a sterile 21 gauge syringe needle and dialysed at room temperature for 2 hr in 1,000 volumes of phage buffer. Bacteriophage were then lysed and the DNA isolated as described in Section II.17.A. Further purification of the DNA on Sepharose CL-4B columns was not necessary.

C. Large scale preparation of plasmid DNA

Plasmid DNA was prepared using a method modified from that of

Birnboim and Doly (1979). *E.coli* strain TG-2 cells containing plasmid were inoculated into 200 ml of L-broth containing 50 μg/ml ampicillin. Cultures were incubated at 37°C overnight with shaking. The cells were harvested by centrifugation at 5,000 x g for 10 min and then resuspended in 4 ml of solution A:

25 mM	Tris/HCl, pH 8.0
10 mM	EDTA
0.9% (w/v)	α-D-glucose

Lysozyme was added to a final concentration of 2 mg/ml and the solution incubated on ice for 10 min. To the mixture was added 8 ml of solution B:

1% (w/v)	SDS
0.8% (w/v)	NaOH

Following a further incubation on ice for 10 min, 6 ml of solution C was added:

3 M	sodium acetate
2 M	acetic acid

The solution was mixed by gentle inversion, incubated on ice for 10 min and then centrifuged at 20,000 x g for 20 min (4°C) using a swing-out rotor. The supernatant was retained and extracted with an equal volume of phenol:chloroform [1:1 (v/v)]. Following centrifugation at 4,000 x g for 10 min, the DNA was precipitated from the aqueous phase by addition of 2 volumes ice-cold ethanol and incubation at -20°C for 1 hr.

DNA was recovered by centrifugation at 20,000 x g for 20 min (4°C). The DNA pellet was washed twice with ice-cold 70% (v/v) ethanol, dried under vacuum and resuspended in 8 ml TE buffer containing 1.0 g/ml CsCl. Ethidium bromide was added to a final concentration of 700 μg/ml and the solution centrifuged at 190,000 x g for 40 hr (20°C). The supercoiled DNA band was removed using a sterile 21 gauge syringe needle. Ethidium bromide was removed by repeated gentle extraction of the DNA solution with water-saturated 1-butanol. Excess CsCl was removed by dialysis in 1,000 volumes TE buffer for 2 hr at room temperature. The purified plasmid DNA was precipitated by addition of 0.1 volumes 3 M sodium acetate pH 6.0 and 2 volumes ice-cold ethanol. Following incubation at -20°C for 1 hr, the DNA was recovered by centrifugation as before. The DNA pellet was dried under vacuum and resuspended in 400 μl TE buffer.

D. Small scale preparation of plasmid DNA

Plasmid DNA was prepared on a small scale from 5 ml culture volume by a modification of the method described in Section II.17.C. Cells were firstly pelleted and lysed as for large scale preparations, using 100 μl solution A, 200 μl solution B and 150 μl solution C.

The crude DNA pellet was resuspended in 250 μl TE buffer and purified further by ammonium acetate precipitation according to Agellon and Chen (1986). To the DNA solution was added 0.5 volumes of 7.5 M ammonium acetate. Following incubation on ice for 15 min, the samples were centrifuged at 12,000 x g for 15 min to pellet the RNA. To the supernatant was added 2 volumes ice-cold ethanol and this was incubated on ice for a further 20 min to precipitate plasmid DNA. The DNA was recovered by centrifugation as before, washed with 70% (v/v) ice-cold ethanol, dried under vacuum and resuspended in 20 µl TE buffer. DNA prepared in this way was a suitable template for DNA sequencing reactions.

E. Preparation of high molecular weight DNA from plants

High molecular weight DNA was isolated from the expanding first leaves of wheat seedlings grown as described in Section II.2. The method used was a modification of that described by Murray and Thompson (1980). Briefly, 20 g of leaves frozen in liquid nitrogen were ground to a fine powder using a mortar and pestle. The tissue was transferred to 120 ml boiling CTAB extraction buffer:

50 mM	Tris/HCl, pH 8.0
10 mM	EDTA
700 mM	NaCl
1% (w/v)	CTAB
1% (v/v)	2-mercaptoethanol

The solution was mixed gently and incubated at 55°C for 30 min. The mixture was gently extracted with an equal volume of chloroform:isoamyl alcohol [24:1 (v/v)] and centrifuged at 12,000 x g for 10 min (20°C). The aqueous phase was recovered using a wide-bore pipette and DNA precipitated from the aqueous phase by addition of an equal volume of CTAB precipitation buffer:

50 mM	Tris/HCl, pH 8.0
10 mM	EDTA
1% (w/v)	CTAB
1% (v/v)	2-mercaptoethanol

Following incubation at room temperature for 30 min, the DNA was pelleted by centrifugation at 4,000 x g for 10 min (4°C) and resuspended in 70 ml TE buffer containing 1 g/ml CsCl and 700 μg/ml ethidium bromide. Equilibrium density gradient centrifugation and subsequent treatment of the DNA was performed as described in Section II.17.C. Extra care was taken to prevent shearing of the DNA.

F. Estimation of DNA quality and quantity

The amount of DNA present in samples was calculated by measuring the ultraviolet absorbance at 260 nm in a Pye Unicam Model SP8-400 spectrophotometer. A DNA solution with a concentration of 50 μg/ml was taken as having an absorbance of 1.0 in a 1.0 cm path length.

The quality of DNA was assessed by the profile obtained after electrophoresis on agarose gels (see Section II.18.A) before and after digestion with restriction endonucleases (see Section II.19).

18. AGAROSE GEL ELECTROPHORESIS OF NUCLEIC ACIDS

A. Agarose gel electrophoresis of DNA

DNA was analysed on agarose gels ranging from 0.5% (w/v) to 1.2% (w/v) agarose depending on the size of the DNA to be fractionated (Maniatis *et al*,1982). Generally high molecular weight plant or bacteriophage DNA was separated on a 0.5% (w/v) agarose gel. Plasmid DNA or restriction endonuclease-digested DNA was separated on a 0.7% (w/v) agarose gel. For the analysis of size-fractionated cDNAs as described in Section II.14.E, a 1.2% (w/v) agarose gel was employed to obtain better resolution (in the region of 500 bp).

For the preparation of agarose gels, the appropriate amount of agarose was melted in 1XTBE buffer:

90 mM	Tris base
90 mM	boric acid
2.4 mM	EDTA

pH 8.3

The gel mix was kept at 65°C until required and then ethidium bromide was added to a final concentration of 1 μg/ml.

Samples were prepared for electrophoresis by addition of 0.2 volumes of 5X DNA sample buffer:

5X	TBE buffer
50% (v/v)	glycerol
0.25% (w/v)	bromophenol blue

Small minigels (7.5 cm x 10 cm x 0.5 cm) employed for the routine analysis of DNA samples were electrophoresed in 1XTBE buffer, at 100 mA constant current for 30 min. Larger gels (20 cm x 20 cm x 0.5 cm) were electrophoresed in 1XTBE buffer, at 30 mA constant current for 20 hr.

The size of DNA molecules was determined by comparison with known size markers. The size markers used were a *Hin*dIII digest of λ DNA containing fragments of 23.13 kb, 9.41 kb, 6.68 kb, 4.36 kb, 2.32 kb, 2.03 kb, 0.56 kb and 0.12 kb in length.

Following electrophoresis, DNA fragments were visualised using an ultraviolet transilluminator at 254 nm wavelength.

B. Electrophoresis of RNA in DMSO/glyoxal gels

For electrophoresis of RNA samples a 1% (w/v) agarose gel (20 cm x 20 cm x 0.5 cm) was prepared in 1XTAE buffer:

12 mM	Tris/HCl, pH 7.0
6 mM	sodium acetate
3 mM	EDTA

Samples were prepared for electrophoresis according to McMaster and Carmichael (1977). Size markers were a *Hin*dIII digest of λ DNA, labelled by end-filling as described in Section II.22.B and denatured by boiling for 90 sec prior to sample preparation. Each sample, and also size markers, were made to the following final concentrations of reagents in a volume of 50 μl:

1X	TAE buffer
20 μg/ml	poly(A)-containing RNA
5% (v/v)	deionised glyoxal
50% (v/v)	recrystallised DMSO

Samples were incubated at 50°C for 20 min and then placed on ice. Immediately prior to electrophoresis, 0.1 volumes of 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue was added to each sample.

Electrophoresis was carried out in 1XTAE buffer with a constant voltage of 5 volt/cm for 3.5 hr. The buffer was continuously recirculated to maintain pH< 8.0.

19. RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction enzyme digestions were routinely carried out in 20-500 μl reaction volumes. The reactions contained the following final concentrations of reagents:

1X	restriction enzyme buffer
100 μg/ml	DNA
10 units/μg DNA	restriction enzyme

The restriction enzyme buffers used were obtained from commercial sources and a list of these buffers is shown in Table II.2. Restriction enzyme digestions were incubated at 37°C for 1-3 hr.

Table II.2 : Components of 10X stock restriction enzyme buffers

	Concentrations of reagents in 10X restriction buffers						
Enzymes	Tris/HCl (mM)	pH	NaCl (mM)	$MgCl_2$ (mM)	2-mercapto ethanol (mM)	BSA %(w/v)	KCl (mM)
*Bam*HI, *Bgl*II, *Eco*RI, *Hin*fI, *Nco*I, *Nde*I, *Pst*I, *Xba*I,	10	7.5	100	7	7	0	0
*Acc*I,*Hin*dIII	10	7.5	60	7	7	0	0
*Kpn*I	6	7.5	6	6	6	0.02	0
*Sst*I	10	7.5	0	7	7	0	0
*Rsa*I	10	8.0	0	7	7	0	20

20. SUB-CLONING OF SPECIFIC DNA FRAGMENTS

Methods employed for the sub-cloning of specific DNA fragments were modifications of those described by Maniatis *et al* (1982).

A. Electroelution of DNA from agarose gels

Fragments required for sub-cloning into plasmid pBSM13+ were recovered from agarose gels by electroelution. Following restriction endonuclease digestion and gel electrophoresis, particular DNA fragments of interest were localised using a long wavelength (300-360 nm) ultraviolet lamp. The DNA bands were excised from the gel using a scalpel blade and placed inside a length of dialysis tubing containing 400 µl of 0.1XTBE buffer. The dialysis tubing was placed into an electrophoresis tank and 240 volts applied for 30 min, or until the DNA could be visualised on the inner wall of the dialysis tubing. The polarity of the current was reversed for 30 sec and the buffer was then recovered. The DNA solution was extracted with an equal volume of phenol:chloroform [1:1 (v/v)] and centrifuged at 12,000 x g for 5 min. The DNA was precipitated from the aqueous phase by addition of 0.1 volumes 3 M sodium acetate, pH 6.0, and 2 volumes of ice-cold ethanol. Following an overnight incubation at -20°C, the DNA was recovered by centrifugation at 12,000 x g for 10 min, washed with ice-cold 70% (v/v) ethanol, dried under vacuum and resuspended in TE buffer to an approximate concentration of 20 ng/ml.

B. Preparation of vector DNA

Vector DNA (pBSM13+; 2 μg) was digested with the appropriate restriction enzyme(s) as described in Section II.19. Following this step, the DNA was phosphatased to eliminate self-ligation events from the ligation reaction. This was achieved by the addition of 10 units of calf intestinal alkaline phosphatase to the restricted DNA solution, followed by incubation at 37°C for 30 min. The vector was deproteinised by extraction with an equal volume of phenol:chloroform [1:1 (v/v)]. Following centrifugation at 12,000 x g for 5 min, the DNA was precipitated from the aqueous phase by addition of 0.1 volumes 3 M sodium acetate, pH6.0, and 2 volumes of ice-cold ethanol. After an overnight incubation at -20°C, the DNA was recovered by centrifugation at 12,000 x g for 10 min and the pellet was washed with ice-cold 70% (v/v) ethanol, dried under vacuum and resuspended to an approximate concentration of 20 ng/ml.

C. Ligation of fragment DNA to vector DNA

Electroeluted DNA fragments were ligated to linearised pBSM13+ DNA in a reaction mixture containing the following final concentrations of reagents:

50 mM	Tris/HCl, pH 7.5
10 mM	$MgCl_2$
10 mM	DTT
1 mM	ATP
2 ng/µl	linear,phosphatased pBSM13+ DNA
1-10 ng/µl	fragment DNA
0.63 units/µl	T_4 DNA ligase

The final volume of the reaction mixture was 10 µl. Reactions were incubated at 15°C overnight.

D. Preparation of transformation-competent *E.coli* strain TG-2 cells

Transformation-competent cells were prepared from a stationary phase culture in L-broth. The stationary phase cells were diluted 1/100 into 20 ml of fresh L-broth and then incubated at 37°C for 2 hr with shaking. The cells were harvested by centrifugation at 1,500 x g for 10 min and resuspended in 5 ml ice-cold 50 mM $CaCl_2$. Following an incubation on ice for 30 min, the cells were harvested by centrifugation as before and resuspended in 1 ml ice-cold 50 mM $CaCl_2$. Transformation-competent cells were kept for a maximum of 24 hr at 4°C.

E. Transformation and plating of competent cells

E.coli strain TG-2 competent cells were transformed with recombinant pBSM13+ DNA by mixing 200 µl of competent cells with

5 μl of ligation mixture. The cells were incubated on ice for 1 hr and then heat-shocked by incubation at 42°C for 5 min. Following this step, 500 μl of L-broth was added to the transformed cells, which were subsequently incubated at 37°C for 1 hr. The transformed cells were spread onto L-plates (see Section II.15.B) containing 50 μg/ml ampicillin, 20 μg/ml IPTG and 80 μg/ml X-gal. The plates were incubated at 37°C overnight.

21. NUCLEIC ACID HYBRIDISATIONS

A. Transfer of RNA onto nylon membranes

Following electrophoresis as described in Section II.18.B, RNA was transferred from agarose gels onto GeneScreen Plus membranes by capillary blotting. The method used was a modification of that described by Southern (1975).

The nylon membrane was soaked for 15 min in transfer buffer (10XSSC solution). The gel was placed onto the transfer apparatus described by Maniatis *et al* (1982). The membrane was placed on top of the gel and then overlaid with a sheet of 3MM filter paper (Whatman) soaked in transfer buffer. Care was taken to ensure that no air bubbles were trapped between the layers of gel, membrane and filter paper. Additional sheets of dry 3MM paper were placed on top of the assembly, followed by 15 cm thickness of absorbant tissues and a 2 kg weight. The capillary blotting was carried out for 16-24 hr at room temperature.

The membrane containing RNA was subsequently washed for 5 min at 100°C in 20 mM Tris/HCl, pH 8.0, to reverse the glyoxalation of the RNA. The filter was then dried at 55°C for 30 min and stored at 4°C in a sealed plastic bag until required for hybridisation experiments.

B. Transfer of DNA onto nylon membranes

Following electrophoresis as described in Section II.18.A, DNA was transferred from agarose gels onto Hybond N membranes by capillary blotting. The method used was a modification of that described by Southern (1975).

The DNA was firstly denatured by soaking the gel for 1 hr in denaturing solution:

1.5 M	NaCl
500 mM	NaOH

Following this, the gel was neutralised by soaking for 45 min in neutralisation solution:

500 mM	Tris/HCl, pH 7.5
1.5 M	NaCl

The DNA was then blotted onto membranes as described in Section II.21.A for RNA blotting, using instead 20XSSC solution as the transfer buffer.

The membrane containing DNA was washed briefly in 5XSSC solution to remove residual agarose. Excess moisture was allowed to evaporate from the membrane at room temperature. DNA was cross-linked to the membrane by exposure for 30 sec to long wavelength (300-360 nm) ultraviolet irradiation at a height of 5 cm. The membrane was then dried completely at 55°C for 1 hr.

C. Hybridisation of DNA to DNA immobilised on nylon membranes

DNA immobilised onto Hybond N membranes was hybridised to nick-translated DNA probes as follows. Membranes were incubated for 4 hr at 65°C with 250 ml of hybridisation buffer:

5X	SSPE solution
0.25% (w/v)	Cadbury's Marvel
0.1% (w/v)	SDS
0.01% (w/v)	denatured herring sperm DNA

Incubations were carried out in sealed plastic bags. Following prehybridisation, the solution was decanted and replaced by 15 ml of hybridisation buffer containing ca. 2 ng/ml denatured probe DNA. The probe had been previously denatured by boiling for 5 min. Hybridisation reactions were carried out at 65°C overnight. Non-specifically hybridised DNA was removed by washing the membranes four times in 2XSSC solution containing 0.1% (w/v) SDS at 65°C. Washes were of 30 min duration. Additional washes in decreasing concentrations of SSC solution were performed as required and the details of these washes are provided with the results of individual experiments (see Section III).

While the washed membranes were still wet they were wrapped in Saran wrap and exposed to X-ray film (Fuji NIF-RX) with an intensifying screen at -70°C.

D. Hybridisation of DNA to RNA immobilised on nylon membranes

RNA immobilised on GeneScreen Plus membranes was hybridised to nick-translated DNA probes using a method modified from that recommended by the supplier of the membrane. Membranes were incubated in sealed plastic bags for 4 hr at 42°C with 25 ml of the following solution:

5X	SSPE solution
50% (v/v)	recrystallised formamide
1% (w/v)	SDS
0.1% (w/v)	polyvinyl pyrrolidone
0.1% (w/v)	bovine serum albumin
0.1% (w/v)	ficoll (mol.wt 400,000)
10.0% (w/v)	dextran sulphate
0.01% (w/v)	denatured herring sperm DNA

Following prehybridisation the solution was decanted and replaced with 10 ml of the same buffer at 42°C. The nick-translated DNA probe was denatured by boiling for 5 min and then added to the plastic bag. Hybridisation reactions were carried out overnight at 42°C. Non-specifically hybridised DNA was removed by washing the membrane with the following series of solutions:

1. Wash once with 500 ml of 5XSSPE solution containing 0.1% (w/v) SDS, at room temperature for 5 min.

2. Wash twice with 500 ml of 2XSSC solution containing 0.1% (w/v) SDS, at room temperature for 5 min each.
3. Wash twice with 500 ml of 2XSSC solution containing 1.0% (w/v) SDS, at 42°C for 30 min each.
4. Wash twice with 500 ml of 2XSSC solution containing 1.0% (w/v) SDS, at 60°C for 30 min each.
5. Wash twice with 500 ml of 0.1XSSC solution containing 1.0% (w/v) SDS, at room temperature for 30 min each.

The wet membranes were wrapped in Saran wrap and exposed to X-ray film with an intensifying screen at -70°C.

22. RADIO-LABELLING OF DNA

A. Nick-translation of DNA

DNA was nick-translated using a BRL nick-translation kit according to Rigby *et al* (1977). The reaction contained the following final concentrations of reagents in a volume of 50 µl:

50 mM	Tris/HCl, pH 7.8
5 mM	$MgCl_2$
10 mM	2-mercaptoethanol
10 µg/ml	bovine serum albumin
1-2 µg/ml	linear probe DNA
0.2 mM	dATP, dGTP, dTTP (each dNTP)
1 mCi/ml	[α-^{32}P]dCTP (> 400 Ci/mmol)
4 units/ml	DNA polymerase I
0.4 ng/ml	DNase I

The DNA polymerase I and DNase I were the final components added to the reaction mixture. Following incubation at 15°C for 1 hr the reaction was stopped by addition of EDTA to a final concentration of 20 mM. The mixture was extracted with an equal volume of phenol:chloroform [1:1 (v/v)]. Following centrifugation at 12,000 x g for 5 min, the aqueous phase was chromatographed on a 2.2 ml column of Sephadex G-50 fine packed into a Pasteur pipette. The radioactively-labelled DNA was eluted with TE buffer and fractions of 100 µl volume were collected. Fractions corresponding to the first peak of radioactivity were pooled and used in hybridisation experiments.

B. End-filling of DNA

λ DNA digested with restriction enzyme *Hind*III was labelled by filling in the recessed 3'-termini with Klenow fragment of *E.coli* DNA polymerase, according to Maniatis *et al* (1982). The reaction mixture contained the following final concentrations of reagents in a volume of 50 µl:

50 mM	Tris/HCl, pH 7.8
5 mM	MgCl$_2$
10 mM	2-mercaptoethanol
10 µg/ml	bovine serum albumin
100 µg/ml	λ DNA, *Hind*III digest
0.2 mM	dATP, dGTP, dTTP (each dNTP)
1 mCi/ml	$[\alpha\text{-}^{32}\text{P}]$dCTP (> 400 Ci/mmol)
0.5 units/µl	Klenow fragment

The reaction mixture was incubated at 37°C for 30 min. Termination of the reaction, deproteinisation and chromatography of the labelled DNA was carried out as described in Section II.22.A. The labelled DNA size markers were stored at -20°C.

23 CELL-FREE SYNTHESIS OF ADP-GLUCOSE PYROPHOSPHORYLASE mRNA

Plasmid WE:AGA.7 contains the cDNA insert encoding wheat endosperm ADP-glucose pyrophosphorylase, sub-cloned into plasmid pBSM13+. The cDNA was transcribed *in vitro* using a cell-free transcription kit supplied by Stratagene Cloning Systems. Plasmid WE:AGA.7 DNA was linearised by digestion with restriction endonuclease *Xba*I as described in Section II.19. Proteinase K was added to a final concentration of 200 μg/ml and this mixture incubated at 37°C for 30 min. The DNA solution was deproteinised by extraction with an equal volume of phenol:chloroform [1:1 (v/v)]. The linearised DNA was precipitated from the aqueous phase by addition of 0.1 volumes 3 M sodium acetate, pH 6.0, and 2 volumes ice-cold ethanol. Following incubation at -20°C overnight, the DNA was recovered by centrifugation at 12,000 x g for 10 min. The pellet was washed with ice-cold 70% ethanol, dried under vacuum and resuspended in sterile double-distilled water to a final concentration of 500 μg/ml.

The transcription reaction was carried out in sterile Eppendorf microcentrifuge tubes and contained the following final concentrations of reagents:

50 μg/ml	*Xba*I cut WE:AGA.7 DNA
40 mM	Tris/HCl, pH 8.0
8 mM	$MgCl_2$
50 mM	NaCl
500 μM	ATP, CTP, GTP, UTP mix
10 mM	DTT
2 mM	spermidine
500 units/ml	T_7 RNA polymerase

The reaction volume was 100 μl. Following an incubation at 37°C for 2 hr, the DNA template was digested by addition of RNase-free DNase to a final concentration of 50 units/ml and further incubation at 37°C for 10 min. The RNA solution was deproteinised and precipitated as described above. The RNA was recovered by centrifugation at 12,000 x g for 10 min, washed twice with ice-cold 80% (v/v) ethanol, dried under vacuum and resuspended in 10 μl of sterile double-distilled water. RNA was translated *in vitro* as described in Section II.13. The *in vitro*-synthesised polypeptides were subsequently precipitated by anti-spinach leaf ADP-glucose pyrophosphorylase serum as described in Section II.10.B.

24. SEQUENCING OF PLASMID DNA

DNA fragments sub-cloned into plasmid pBSM13+ were sequenced using a ^{35}S-DNA sequencing kit supplied by Stratgene Cloning Systems. The method used was a modification of that recommended by the supplier and is based on the dideoxy method of Sanger *et al*

(1977).

A. Annealing of oligonucleotide primer to template DNA

For each set of sequencing reactions, plasmid DNA was chemically denatured by incubation for 5 min at room temperature in a solution with the following final concentrations of reagents:

200 μg/ml	plasmid DNA
200 mM	NaOH
200 μM	EDTA

The volume of the solution was 10 μl. The denatured DNA solution was placed on ice and the volume adjusted to 30 μl by addition of sterile double-distilled water. DNA was precipitated by addition of 0.1 volumes 3 M sodium acetate, pH 6.0, and an equal volume of ice-cold isopropanol. Following an incubation on ice for 10 min, the DNA was recovered by centrifugation at 12,000 x g for 10 min. The DNA pellet was dried under vacuum for 5 min and resuspended in 9.5 μl of sterile double-distilled water.

The priming reaction was carried out in a total volume of 12 μl with components at the following final concentrations:

100 mM	Tris/HCl, pH 7.5
10 mM	$MgCl_2$
10 mM	DTT
2 mM	EDTA
167 μg/ml	denatured plasmid DNA
4 μg/ml	oligonucleotide primer

The solution was boiled for 1 min and placed immediately at 37°C for 20 min.

B. Sequencing reaction protocol

The annealed template-primer mixture was used immediately as a substrate for sequencing reactions. The volume of the annealing mixture was adjusted to 16 μl by the addition of 2 μl [α-^{35}S]dATP (10 μCi/μl; > 1000 Ci/mmol), 1 μl Klenow fragment (5 units/μl) and 1 μl *E.coli* single-stranded binding protein (0.6 μg/μl).

Sequencing reactions were carried out in four tubes labelled either A,C,G,or T, and containing 2 μl of the corresponding deoxynucleotide/ dideoxynucleotide mixture (dNTP/ddNTP mix A,C,G, or T; see Table II.3) plus 3 μl of supplemented annealing mixture. Following incubation at 37°C for 20 min, 1 μl of dNTP chase solution (10 mM each of dATP, dCTP, dGTP and dTTP) was added to each reaction. The reactions were then incubated for a further 15 min at 37°C. The reactions were terminated by addition of 6 μl formamide dye mixture:

20 mM	EDTA
100% (v/v)	recrystallised formamide
0.1% (w/v)	xylene cyanol
0.1% (w/v)	bromophenol blue

Sequencing reactions were analysed on polyacrylamide gels as described in Section II.24.C.

Table II.3 : Composition of stock deoxynucleotide/dideoxynucleotide mixtures for sequencing reactions.

dNTP or ddNTP Reagent	Concentrations of reagents (μM)			
	A mix	C mix	G mix	T mix
ddATP	5	0	0	0
ddCTP	0	39	0	0
ddGTP	0	0	78	0
ddTTP	0	0	0	241
dCTP	125	6.2	123	120
dGTP	125	123	6.1	120
dTTP	125	123	123	6

Table II.3 : Composition of stock deoxynucleotide/dideoxynucleotide mixtures for sequencing reactions.

dNTP or ddNTP Reagent	Concentrations of reagents (μM)			
	A mix	C mix	G mix	T mix
ddATP	5	0	0	0
ddCTP	0	39	0	0
ddGTP	0	0	78	0
ddTTP	0	0	0	241
dCTP	125	6.2	123	120
dGTP	125	123	6.1	120
dTTP	125	123	123	6

C. Electrophoresis on polyacrylamide sequencing gels

Sequencing reactions were analysed on polyacrylamide gels (39 cm x 30 cm x 0.2 mm). Gels were poured between two silanised glass plates and contained the following final concentrations of reagents:

5.7% (w/v)	acrylamide
0.3% (w/v)	bisacrylamide
7 M	urea
133 mM	Tris base
44 mM	boric acid
3 mM	EDTA
0.1% (v/v)	TEMED
0.06% (w/v)	ammonium persulphate

pH 8.8

The gel mix was allowed to polymerise for at least 1 hr at room temperature.

The electrophoresis running buffer was 1X sequencing TBE buffer:

133 mM	Tris base
44 mM	boric acid
3 mM	EDTA

pH 8.8

For electrophoresis of sequencing reaction mixtures, samples were boiled for 3 min and 2 μl aliquots immediately loaded onto a polyacrylamide sequencing gel. Electrophoresis was carried out at

50 W constant power for 3 hr or 5 hr. The gel was fixed in 10% (v/v) acetic acid for 15 min at room temperature and then rinsed briefly with distilled water. Excess moisture was removed from the surface of the gel by blotting with tissues. The gel was then transferred onto 3MM filter paper (Whatman), dried under vacuum at 80°C for at least 30 min and then exposed to X-ray film (Fuji NIF-RX) overnight at room temperature. Sequence data was analysed by the computer programmes of Staden (1982) and Devereux *et al* (1987).

SECTION III- RESULTS AND DISCUSSION

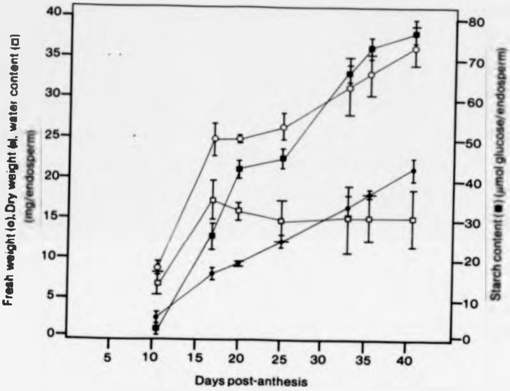
1. CHARACTERISATION OF WHEAT ENDOSPERM DEVELOPMENT

A. Developmental changes in fresh weight, dry weight, water content and starch content

The starch content of endosperm was measured over the period from 11 d.p.a. to 40 d.p.a.. Endosperms of various ages were dissected from grain which were removed from the mid-spike position of wheat ears. The samples were weighed and then half of the samples taken at each time-point were assayed for starch content as described in Section II.5; the remaining half of the samples were dried at 80°C for 48 hr and the dry weights were determined. The water content of endosperm samples was determined as the difference between fresh weight and dry weight measurements. For all measurements, triplicate samples, each consisting of 25 endosperms, were prepared. The results are expressed on a 'per endosperm' basis. Measurements of the mitotic index of wheat endosperms from two cultivars (*Triticum aestivum* L. Chinese Spring and *Triticum aestivum* L. Spica) indicate that endosperm cell division ceases between 15-20 d.p.a. (Chojecki, 1986). Thus, the results expressed on a 'per endosperm' basis should correlate with those obtained if expressed on a 'per cell' basis, at least for the greater part of this experiment.

Fresh weight and dry weight of wheat endosperms increased throughout the period analysed, as shown in Figure III.1. The most rapid rate of increase in endosperm fresh weight occurred between 11 d.p.a. and 17 d.p.a.. There was a definite lag in fresh weight increase shortly thereafter, continuing up to 25 d.p.a.. During the same period the dry weight continued to increase, although at

Figure III.1: Changes in fresh weight, dry weight, water content and starch content of wheat endosperm during development. Each point represents the mean of three replicate samples. Vertical bars represent the standard error of the mean.


Fresh weight (o), Dry weight (●), water content (□)
(mg/endosperm)
0
5
10
15
20
25
30
35
40
Starch content (■) (μmol glucose/endosperm)
0
10
20
30
40
50
60
70
80
5
10
15
20
25
30
35
40
Days post-anthesis

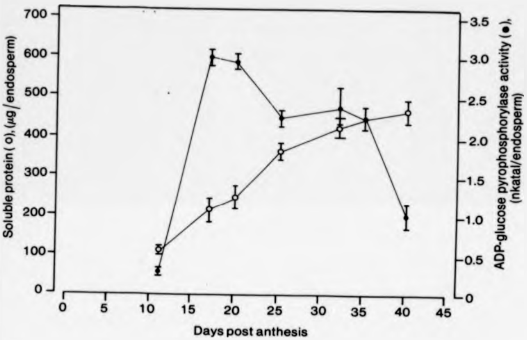
a lower rate than fresh weight. After 25 d.p.a., the fresh weight and dry weight of endosperms increased at the same rate. Water content increased until 17 d.p.a. and thereafter remained approximately constant. The percentage of endosperm fresh weight attributable to water content actually declined at a constant rate from 80% (w/w) at 11 d.p.a., to 40% (w/w) at 40 d.p.a..

The increase in dry weight of wheat endosperms was accompanied by increased starch content throughout the period 11-40 d.p.a. (Figure III.1). The most rapid rise in starch content of endosperm was observed between 11 d.p.a. and 20 d.p.a.. This rise was followed immediately by a lag phase in starch accumulation between 20 d.p.a. and 25 d.p.a.. A constant, high rate of starch deposition occurred between 25 d.p.a. and 35 d.p.a., after which time the rate slowed once more. The rounded appearance of endosperms at 40 d.p.a., and the decline in the rate of starch accumulation during the period following 35 d.p.a. suggested that the grain-filling period was nearing completion at this time. The results obtained in this study are compatible with data on whole wheat grain, described by Turner (1969).

B. Developmental changes in ADP-glucose pyrophosphorylase activity

Wheat grain were removed from the mid-spike position of ears at various stages of development, from 11 d.p.a. to 40 d.p.a.. For each time-point, endosperm were dissected free of pericarp and aleurone layers and assayed in triplicate for ADP-glucose pyrophosphorylase activity (Section II.3.A.1) and soluble protein content (Section II.4.). The results are presented in Figure III.2

Figure III.2: Changes in ADP-glucose pyrophosphorylase activity and soluble protein content of wheat endosperm during development. Each point represents the mean of three replicate samples. Vertical bars represent the standard error of the mean.


Soluble protein (o), (µg / endosperm)
700
600
500
400
300
200
100
0
ADP-glucose pyrophosphorylase activity (●), (nkatal/endosperm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
30
35
40
45
Days post anthesis

and agree with those of Turner (1969). The protein content per endosperm increased throughout development, with the fastest rate of soluble protein accumulation occurring in the first 25 days post anthesis. The activity of ADP-glucose pyrophosphorylase increased 10-fold from 0.3 nkatal/endosperm at 11 d.p.a., to 3 nkatal/endosperm at 17 d.p.a.. In the period between 17-20 d.p.a., the ADP-glucose pyrophosphorylase activity was maximal at approximately 3 nkatal/endosperm (Figure III.2). After 20 d.p.a., the level of enzyme activity declined to 2.25 nkatal/endosperm and remained approximately constant for a further 10 days. Finally, between 35 d.p.a. and 40 d.p.a. the level of enzyme activity per endosperm declined to only 0.9 nkatal/endosperm i.e. 30% of the maximal value.

C. Discussion

Wheat endosperm development can be divided into three distinct phases i.e. mitosis, cell expansion and maturation drying (Bechtel *et al*, 1982; Briarty *et al*, 1979). Initially, the endosperm forms as a multinucleated syncytium, which undergoes a process of cellularisation and mitosis during the first developmental phase. The total DNA content of wheat endosperms increases until approximately 24 d.p.a. as a consequence of both cell division and endoreduplication (Chojecki, 1981; Chojecki, 1986). However, measurements of the mitotic index of wheat endosperms indicates that cell division actually ceases earlier than this, at between 15-20 d.p.a. (Chojecki, 1986). The exact time at which this phase of endosperm development ceases depends on both the genetic background of the wheat cultivar and the environment in which the plant is grown (P. Keeling, I.C.I. Plant Biotechnology Group, Runcorn, Cheshire, U.K.; personal communications). In the current study, the fresh weight, dry weight, starch content and water content of wheat endosperm increased most dramatically in the period between 11 d.p.a. and 17 d.p.a.. The magnitude of the observed increases in these parameters during the period between 11-20 d.p.a. is probably the result, at least in part, of the cell division process. As a consequence, the intracellular levels of starch, protein and water may have been more or less constant, or increased only slightly during this period. If so, then the apparent lag in starch accumulation between 20-25 d.p.a. may have been the result of reduced cell division at this time, prior to a rapid increase in intracellular starch levels after 25 d.p.a..

During the middle phase of wheat endosperm development, storage proteins and starch accumulate (Bechtel *et al*, 1982; Briarty *et al*, 1979). As shown in Figure III.1, starch levels increased throughout the period from 20-40 d.p.a., but the rate of accumulation of starch was lower in the later period from 35-40 d.p.a., than during the earlier developmental stages. As the grain appeared quite round and full of starch by 35 d.p.a., it is reasonable to assume that the starch accumulation period was nearing an end.

In the current investigation, it is unlikely that the the developmental time-course extended into the maturation-drying phase of development (phase 3, Bechtel *et al*, 1982; Briarty *et al*, 1979), since the water content of endosperm was observed to be constant throughout the sampling period. Although the percentage water content of endosperms fell from 80% (w/w) at 11 d.p.a. to

40% (w/w) at 40 d.p.a., Turner (1969) has calculated that the water content of a mature wheat grain accounts for only 12% (w/w) of the total grain fresh weight.

ADP-glucose pyrophosphorylase activity per endosperm also rose rapidly during the period from 11-17 d.p.a. (see Figure III.2). As cell division was probably taking place at the same time, the magnitude of the increase in enzyme activity per cell is likely to be less than that observed for the endosperm as a whole. In potato tubers also, the initial increase in ADP-glucose pyrophosphorylase activity coincides with the procambial cellular division phase of the tuberisation process (Reeve *et al*, 1969). On the basis of the observed developmental change in enzyme activity, samples were routinely harvested at 20-21 d.p.a. for protein purification experiments, when ADP-glucose pyrophosphorylase activity per endosperm (or per cell) was maximal. Since the initial rapid increase in ADP-glucose pyrophosphorylase activity (i.e. between 11-17 d.p.a.) probably coincided with the cell division phase of development, it seemed likely that mRNA encoding the enzyme would be most abundant in the same period. At every time later than 17 d.p.a., ADP-glucose pyrophosphorylase activity was either constant or falling, suggesting that the amount of ADP-glucose pyrophosphorylase mRNA in endosperms of this age was less than maximal. Thus, in experiments requiring wheat endosperm mRNA, grain were harvested at 14-15 d.p.a..

When this developmental study was first carried out, it was envisaged that cDNAs encoding ADP-glucose pyrophosphorylase would be produced and used to determine if the developmental pattern of enzyme activity was regulated at the level of transcription. However, before a cDNA encoding wheat endosperm ADP-glucose pyrophosphorylase was characterised, Krishnan *et al* (1986) successfully isolated a cDNA encoding the rice endosperm enzyme. Reeves *et al* (1986) then used this rice endosperm cDNA in Northern blot experiments to measure amounts of wheat endosperm ADP-glucose pyrophosphorylase mRNA during development; at the same time, Western blot analysis using anti-spinach leaf ADP-glucose pyrophosphorylase serum revealed the presence of ADP-glucose pyrophosphorylase polypeptides at every developmental stage from 6 d.p.a. to 31 d.p.a.. Reeves *et al* (1986) found that the accumulation profile of ADP-glucose pyrophosphorylase mRNA follows both the corresponding polypeptide accumulation profile and the enzyme activity profile. In the first 10 d.p.a., the amount of mRNA encoding ADP-glucose pyrophosphorylase was shown to increase to 50% of the maximal level, which was attained at 14 d.p.a.. The peak in amount of ADP-glucose pyrophosphorylase mRNA preceded the accumulation of maximum amounts of ADP-glucose pyrophosphorylase polypeptide by 4 days. In addition, the decline in enzyme activity observed in the period after 20 d.p.a. was shown by Reeves *et al* (1986) to be accompanied by a reduction in mRNA amounts. This result suggests that the change in ADP-glucose pyrophosphorylase activity in wheat endosperm is regulated primarily at the level of transcription. In view of this report, observations on the developmental regulation of wheat endosperm ADP-glucose pyrophosphorylase were not made during the present study. Instead, the emphasis was placed on the isolation and characterisation of cDNAs encoding organ-specific isoenzymes of wheat ADP-glucose pyrophosphorylase.

2. PURIFICATION OF WHEAT ENDOSPERM ADP-GLUCOSE PYROPHOSPHORYLASE

A. Purification of protein for immunisation of rabbits

ADP-glucose pyrophosphorylase was partially purified according to purification protocol 1, as described in Section II.6.B. The purification consisted of two steps, DEAE—Sepharose chromatography and molecular sieving through Superose 12 HR 10/30. Typical purification results are presented in Table III.1. Putative ADP-glucose pyrophosphorylase polypeptides were subsequently purified from SDS/polyacrylamide gels as described in Section II.6.D.

Following centrifugation of the crude endosperm extract and dialysis of the 39,000 x g supernatant, extracts routinely contained ADP-glucose pyrophosphorylase at a specific activity of 3.6-5.9 nkat/mg protein (average specific activity of 4.8 nkat/mg protein). The enzyme was purified 54- to 79-fold relative to the dialysed 39,000 x g supernatant, with an overall recovery of 10-18%.

The low recovery of ADP-glucose pyrophosphorylase activity suggested that the enzyme was destabilised during one or more stages of the purification. Only 30% of the enzyme activity was recovered following ion-exchange chromatography (Table III.1), suggesting that the enzyme may not be stable in high salt concentrations. In addition, the long cycle-time involved for low pressure chromatographic purifications may have been partially responsible for the poor recoveries from DEAE-Sepharose columns, since the enzyme would have been exposed to contaminating proteases for long periods of time. Following gel filtration, only

Table III.1 : A typical purification of ADP-glucose pyrophosphorylase from wheat endosperm using protocol 1.

Purification steps	Volume (ml)	Protein (mg/ml)	Activity (nkat/ml)	Total activity (nkat)	Yield (%)	Specific activity (nkat/mg)	Purification (-fold)
Dialysed supernatant	9.0	5.00	26.9	242.2	100	5.38	1.0
DEAE-Sepharose	5.0	0.122	15.26	76.3	31.5	125.08	23.2
Concentrated DEAE-Sepharose	0.48	1.268	56.40	27.1	11.2	44.47	8.2
Superose 12 HR 10/30	1.0	0.075	26.2	26.2	10.8	349.33	64.9

30% of the enzyme activity present in the DEAE-Sepharose eluant was recoverable (Table III.1). It was subsequently observed that concentration of the enzyme preparation prior to gel filtration produced a reduction in ADP-glucose pyrophosphorylase activity of up to 70% (Table III.1.). This effect of was observed regardless of the method used to concentrate protein samples (for example ammonium sulfate precipitation, ultrafiltration, osmosis against PEG_{8000}) and could not be reversed by extensive dialysis of the concentrated enzyme preparation.

Elution of ADP-glucose pyrophosphorylase from DEAE-Sepharose produced the elution pattern shown in Figure III.3. The enzyme eluted in a single peak of activity between fractions 98 and 110, with maximum activity found in fraction 102. The conductivity of the peak fraction was measured and corresponded to 170 mM KCl. Fractions 98-105 inclusive were analysed on an SDS/polyacrylamide gel (Figure III.4). Several polypeptides were enhanced in these fractions relative to the crude endosperm extract. The abundance of three polypeptides in the SDS/polyacrylamide gel electrophoretic patterns of fractions correlated with the elution profile of ADP-glucose pyrophosphorylase activity from DEAE-Sepharose columns. This suggests that one, or more of these polypeptides might be ADP-glucose pyrophosphorylase. The putative ADP-glucose pyrophosphorylase polypeptides have apparent molecular weights of 51,000, 50,000 and 49,000. The most abundant polypeptide in fractions containing ADP-glucose pyrophosphorylase activity had a molecular weight of approximately 60,000; however, the elution profile for this protein did not correspond to the enzyme activity elution profile (compare Figures III.3 and III.4).

Chromatography of the partially purified enzyme preparation on

Figure III.3: Chromatography of ADP-glucose pyrophosphorylase on DEAE-Sepharose. Sample loading: 242 nkat ADP-glucose pyrophosphorylase in a crude extract of wheat endosperm. Flow rate: 0.3 ml/min. Fraction volume: 1 ml. The column was washed with 10 mM potassium phosphate buffer (A), followed by 30 mM potassium phosphate buffer (B) and finally with 50 mM potassium phosphate buffer (C). A linear gradient of 0-500 mM KCl was begun at the time indicated by the arrow. (○), ADP-glucose pyrophosphorylase activity; (●), absorbance at 280 nm; (- - -), conductivity.

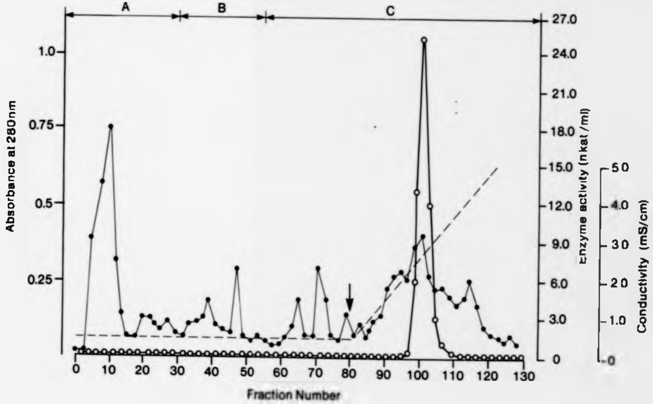
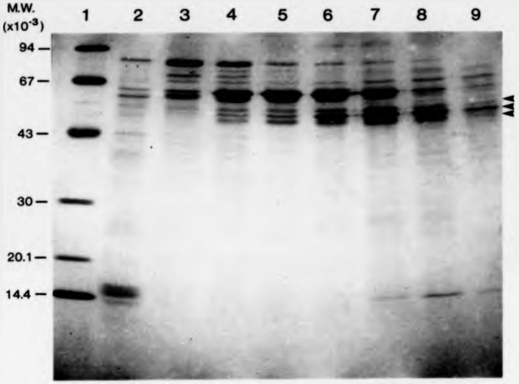

A
B
C
Absorbance at 280nm
1.0
0.75
0.5
0.25
Enzyme activity (n kat / ml)
27.0
24.0
21.0
18.0
15.0
12.0
9.0
6.0
3.0
0
Conductivity (mS/cm)
5 0
4.0
3 0
2 0
1.0
0
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Fraction Number

Figure III.4: Analysis of DEAE-Sepharose column fractions by SDS/polyacrylamide gel electrophoresis. Aliquots of DEAE-Sepharose fractions containing ADP-glucose pyrophosphorylase activity were analysed on a 10-20% (w/v) SDS/polyacrylamide gel as described in the 'Materials and Methods' section. Samples contain approximately 25 μg protein per lane. Lane 1 contains low molecular weight marker proteins. Lane 2 contains ADP-glucose pyrophosphorylase in a crude extract of wheat endosperm. Lanes 3-9 inclusive contain DEAE-Sepharose fractions 98-104 inclusive. Arrows indicate the positions of putative ADP-glucose pyrophosphorylase polypeptides. Molecular weights are indicated on the left.


M.W.
(x10⁻³)
1
2
3
4
5
6
7
8
9
94
67
43
30
20.1
14.4

Superose 12 HR 10/30 produced the elution profile shown in Figure III.5. The ADP-glucose pyrophosphorylase activity eluted in fractions 19-24, immediately prior to the main protein peak. Maximum enzyme activity was found in fraction 22 (Figure III.5). Fractions 19-26 inclusive were analysed on an SDS/polyacrylamide gel (Figure III.6). The elution profile of the putative ADP-glucose pyrophosphorylase polypeptides again matched the ADP-glucose pyrophosphorylase specific activity of eluant fractions (compare Figure III.5 and Figure III.6). The putative ADP-glucose pyrophosphorylase polypeptides were the most abundant protein components of the fraction containing maximum ADP-glucose pyrophosphorylase activity (i.e. fraction 22; see Figure III.6, lane 5). The elution profile of other proteins from the Superose 12 HR 10/30 column did not correspond to the elution profile of ADP-glucose pyrophosphorylase. In particular, the 60 kD polypeptide which was present in abundance following DEAE-Sepharose chromatography, was depleted in extracts following gel filtration.

The two-step chromatographic purification of ADP-glucose pyrophosphorylase was followed by SDS/polyacrylamide gel electrophoresis of the pooled extracts obtained at each purification step (Figure III.7). The enzyme was not electrophoretically homogeneous following the gel filtration step. However, the putative ADP-glucose pyrophosphorylase polypeptides comprised the major protein content of the partially purified enzyme preparation. These proteins were also specifically enhanced at each stage of the purification, as a proportion of the total protein content of samples (Figure III.7). Laser densitometry of the polypeptide profile of partially purified ADP-glucose

Figure III.5: Chromatography of ADP-glucose pyrophosphorylase on Superose 12 HR 10/30. Sample loading: 27.1 nkat ADP-glucose pyrophosphorylase in extract after DEAE-Sepharose step. Flow rate: 0.5 ml/min. Fraction volume: 0.5 ml. (○), ADP-glucose pyrophosphorylase activity; (●) absorbance at 280 nm.

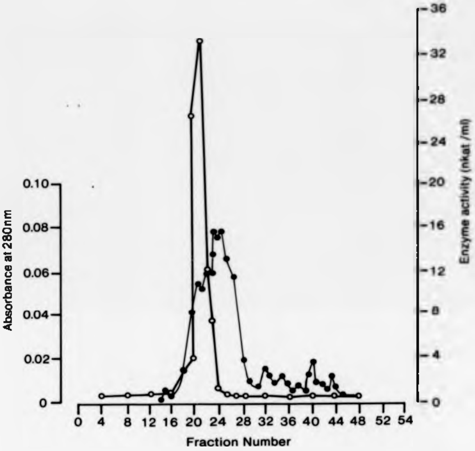

Absorbance at 280nm
0.10
0.08
0.06
0.04
0.02
0
Enzyme activity (nkat /ml)
36
32
28
24
20
16
12
8
4
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
54
Fraction Number

Figure III.6: Analysis of Superose 12 HR 10/30 column fractions by SDS/polyacrylamide gel electrophoresis. Aliquots of Superose 12 HR 10/30 fractions containing ADP-glucose pyrophosphorylase activity were analysed on a 10-20% (w/v) SDS/polyacrylamide gel as described in the 'Materials and Methods' section. Samples contain approximately 25 μg protein per lane. Lane 1 contains low molecular weight protein markers. Lanes 2-9 inclusive contain 25 μg protein each, from Superose 12 HR 10/30 fractions 19-26 inclusive. Arrows indicate the positions of putative ADP-glucose pyrophosphorylase polypeptides. Molecular weights are indicated on the left.

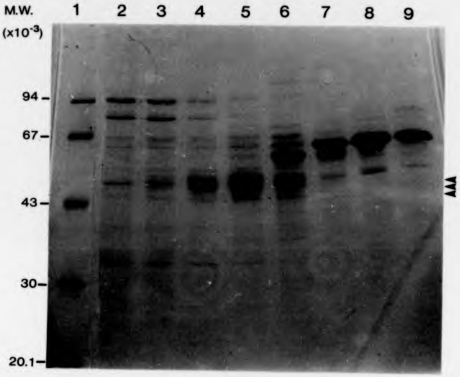

M.W.
(x10^-3)
1
2
3
4
5
6
7
8
9
94
67
43
30
20.1

Figure III.7: Purification of wheat endosperm ADP-glucose pyrophosphorylase using protocol 1. Pooled aliquots from DEAE-Sepharose and Superose 12 HR 10/30 columns containing ADP-glucose pyrophosphorylase activity were analysed on a 10-20% (w/v) SDS/polyacrylamide gel as described in the 'Materials and Methods' section. Samples contain approximately 25 μg protein per lane. Lane 1, crude extract of wheat endosperm (ADP-glucose pyrophosphorylase specific activity 5.38 nkat/mg protein). Lane 2, extract following chromatography on DEAE-Sepharose (ADP-glucose pyrophosphorylase specific activity 44.47 nkat/mg protein). Lane 3, extract following the Superose 12 HR 10/30 step (ADP-glucose pyrophosphorylase specific activity 349.33 nkat/mg protein). Arrows indicate the positions of putative ADP-glucose pyrophosphorylase polypeptides. Molecular weights are indicated on the left.

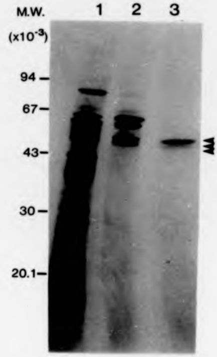
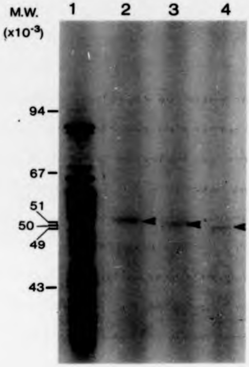

M.W.
(x10⁻³)
1
2
3
94
67
43
30
20.1

Figure III.8: Analysis of putative ADP-glucose pyrophosphorylase polypeptides by SDS/polyacrylamide gel electrophoresis. Electroeluted polypeptides were electrophoresed on a 10-20% (w/v) SDS/polyacrylamide gel as described in the 'Materials and Methods' section. The gel was silver-stained using a Bio-Rad silver stain reagent kit as recommended by the supplier. Lane 1 contains 25 μg protein in a crude extract of wheat endosperm. Lane 2 contains the 51 kD ADP-glucose pyrophosphorylase polypeptide. Lane 3 contains the 50 kD ADP-glucose pyrophosphorylase polypeptide. Lane 4 contains the 49 kD ADP-glucose pyrophosphorylase polypeptide. Molecular weights are indicated on the left.


M.W.
(x10⁻³)
1
2
3
4
94
67
51
50
49
43

pyrophosphorylase indicated that each putative ADP-glucose pyrophosphorylase polypeptide comprised 25-30% of the total protein (result not shown).

The native molecular weight of wheat endosperm ADP-glucose pyrophosphorylase was determined to be 245,000 ± 30,000, by re-chromatography of the partially purified enzyme on Superose 12 HR 10/30. This is consistent with the estimated molecular weight of 260,000 ± 20,000, determined by chromatography of the protein on Sephacryl S-300 sf. In each case, the molecular weight was determined by comparison of the elution volume of ADP-glucose pyrophosphorylase activity with the elution volume of known protein standards, ferritin, catalase and bovine serum albumin.

Sufficient protein to enable the immunisation of rabbits was obtained from 15 individual protein purifications. A total of 1.2 mg protein was obtained from 75 g of wheat endosperm material. The 51 kD, 50 kD and 49 kD putative ADP-glucose pyrophosphorylase polypeptides were subsequently purified by electroelution from polyacrylamide gel slices. The electroeluted proteins were then electrophoresed on an SDS/polyacrylamide gel and appeared to be electrophoretically homogeneous (Figure III.8).

B. Purification of protein for N-terminal amino acid sequence analysis

ADP-glucose pyrophosphorylase activity was partially purified using purification protocol 2, as described in Section II.6.C. The results of a typical purification of enzyme activity are presented in Table III.2. Following centrifugation of the crude extract, the

Table III.2 : A typical purification of ADP-glucose pyrophosphorylase from wheat endosperm using protocol 2.

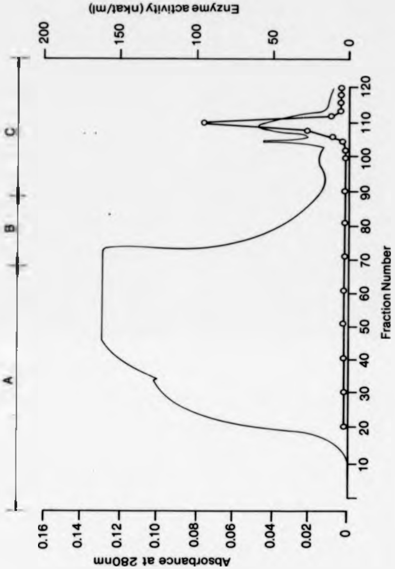
Purification steps	Volume (ml)	Protein (mg/ml)	Activity (nkat/ml)	Total activity (nkat)	Yield (%)	Specific activity (nkat/mg)	Purification (-fold)
39,000 x g supernatant	10.0	7.56	70.99	709.7	100	9.38	1.0
phenyl-Sepharose	11.0	1.427	38.70	425.8	59.9	27.12	2.8
MonoQ HR 5/5	6.0	0.233	34.95	209.7	33.8	150.00	16.1
MonoQ HR 5/5	6.0	0.083	26.88	161.3	22.7	323.85	34.5
Superose 12 HR 10/30	1.0	0.070	48.40	48.4	6.8	691.42	73.7

39,000 x g supernatant contained ADP-glucose pyrophosphorylase at a specific activity of 9.38 nkat/mg protein. This value represents approximately twice the enzyme activity of the same extract in purification protocol 1 (see Table III.1). Throughout purification protocol 1, ADP-glucose pyrophosphorylase was assayed in the direction of synthesis of ADP-glucose as described in Section II.3.A.1. During the present purification, ADP-glucose pyrophosphorylase was assayed in the direction of pyrophosphorylysis, as described in Section II.3.A.2. Thus, the apparent difference in enzyme content of the supernatants obtained in each purification protocol, is probably a reflection of the different Km values of the enzyme for ATP and ADP-glucose. Following purification of ADP-glucose pyrophosphorylase according to purification protocol 2, the enzyme was purified 74-fold relative to the 39,000 x g supernatant (Table III.2). The yield of enzyme activity was 6.8% of the activity found in the 39,000 x g supernatant (Table III.2).

Elution of ADP-glucose pyrophosphorylase from phenyl-Sepharose produced the elution profile shown in Figure III.9. A large proportion of the total protein present in the 39,000 x g supernatant did not bind to phenyl-Sepharose in 500 mM potassium phosphate buffer. ADP-glucose pyrophosphorylase eluted from phenyl-Sepharose in the main protein peak between fractions 103-113, in 10 mM potassium phosphate buffer (Figure III.9). Maximum enzyme activity was found in fraction 110. A 2.8-fold purification of ADP-glucose pyrophosphorylase activity was obtained with phenyl-Sepharose, with nearly 60% recovery of enzyme activity (Table III.2).

Chromatography of ADP-glucose pyrophosphorylase on MonoQ HR 5/5

Figure III.9: Chromatography of ADP-glucose pyrophosphorylase on phenyl-Sepharose. Sample loading: 709.7 nkat ADP-glucose pyrophosphorylase in a crude extract of wheat endosperm. Flow rate: 0.5 ml/min. Fraction volume: 1 ml. The column was washed with 500 mM potassium phosphate buffer (A), followed by 350 mM potassium phosphate buffer (B), and finally the enzyme was eluted with 10 mM potassium phosphate buffer (C). The absorbance at 280 nm was monitored continuously throughout the procedure (unbroken line). (O), ADP-glucose pyrophosphorylase activity.


A
B
C
Absorbance at 280nm
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
Enzyme activity (nkat/ml)
0
50
100
150
200
Fraction Number
10
20
30
40
50
60
70
80
90
100
110
120

provided an additional 8-fold purification of enzyme activity, with approximately 55% recovery (Table III.2). The enzyme eluted between fractions 44-50, with maximum activity found in fraction 46 (Figure III.10). The enzyme began to elute from the column in 200 mM KCl, which is comparable to the reported elution of wheat endosperm ADP-glucose pyrophosphorylase from DEAE-Sepharose in 170 mM KCl (see Section III.2.A).

Re-chromatography of the enzyme preparation on MonoQ HR 5/5 produced an additional 2-fold purification over that obtained previously with this ion-exchange medium (Table III.2). The yield of enzyme activity was nearly 70% of the activity loaded onto the column (Table III.2). The elution profile of ADP-glucose pyrophosphorylase activity during this second cycle on MonoQ HR 5/5 was similar to that obtained previously (see Figure III.11). However, the enzyme eluted in a sharper band of enzyme activity since 90% of the ADP-glucose pyrophosphorylase activity eluted in fractions 44-46 inclusive, compared with only 70% during the first purification cycle on MonoQ HR 5/5.

Following chromatography of the sample on Superose 12 HR 10/30, only 30% of the enzyme activity present in the MonoQ HR 5/5 purified preparation was recovered (Table III.2). This loss in enzyme activity has previously been found to occur during the protein concentration step immediately preceding gel filtration (see Section III.2.A). The elution profile of ADP-glucose pyrophosphorylase activity from Superose 12 HR 10/30 is shown in Figure III.12. The enzyme eluted in the same position as determined previously (see Section III.2.A), with maximum activity found in fraction 22 (compare Figure III.5 and Figure III.12).

Pooled ADP-glucose pyrophosphorylase extracts at each stage of

Figure III.10: Chromatography of ADP-glucose pyrophosphorylase on MonoQ HR 5/5. Sample loading: 425.8 nkat ADP-glucose pyrophosphorylase in extract after phenyl-Sepharose step. Flow rate: 1 ml/min. Fraction volume: 1 ml. A linear gradient of 0-350 mM KCl was begun at the time indicated by the arrow. The absorbance at 280 nm was monitored continuously throughout the procedure (unbroken line). (O), ADP-glucose pyrophosphorylase activity; (- - -), concentration of KCl.

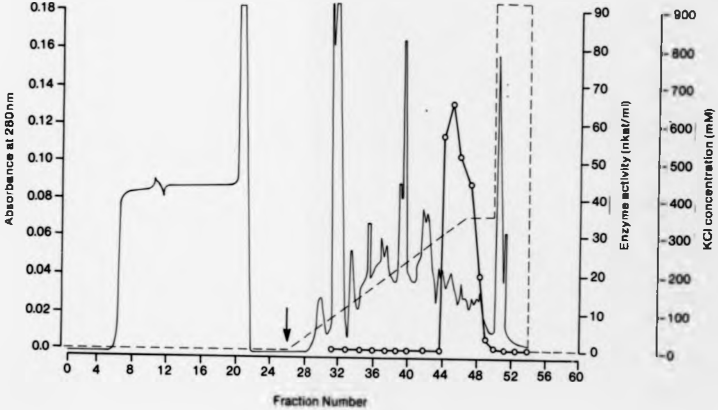

Absorbance at 280nm
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0
Enzyme activity (nkat/ml)
90
80
70
60
50
40
30
20
10
0
KCl concentration (mM)
900
800
700
600
500
400
300
200
100
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Fraction Number

Figure III.11: Re-chromatography of ADP-glucose pyrophosphorylase on MonoQ HR 5/5. Sample loading: 209.7 nkat ADP-glucose pyrophosphorylase in extract after first MonoQ HR 5/5 step. Flow rate: 1 ml/min. fraction volume: 1 ml. A linear gradient of 0-350 mM KCl was begun at the time indicated by the arrow. The absorbance at 280 nm was montiored continuously throughout the procedure (unbroken line). (O),ADP-glucose pyrophosphorylase activity; (---), concentration of KCl.

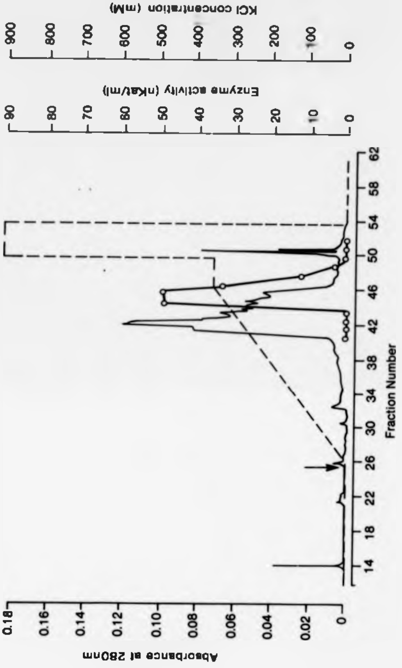

Absorbance at 280nm
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Fraction Number
14
18
22
26
30
34
38
42
46
50
54
58
62
Enzyme activity (nKat/ml)
90
80
70
60
50
40
30
20
10
0
KCl concentration (mM)
900
800
700
600
500
400
300
200
100
0

Figure III.12: Chromatography of ADP-glucose pyrophosphorylase on Superose 12 HR 10/30. Sample loading: 161.3 nkat ADP-glucose pyrophosphorylase in extract after MonoQ HR 5/5 step. Flow rate: 0.5 ml/min. Fraction volume: 0.5 ml. The absorbance at 280 nm was monitored continuously throughout the procedure (unbroken line). (○), ADP-glucose pyrophosphorylase activity.

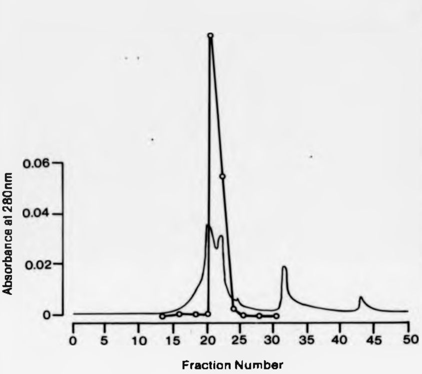
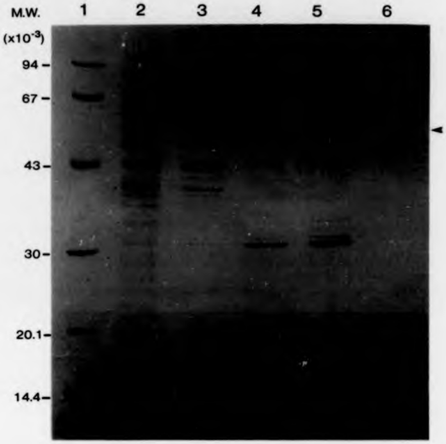

Absorbance at 280nm
0.06
0.04
0.02
0
Enzyme activity (nkat/ml)
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
Fraction Number

Figure III.13: Purification of wheat endosperm ADP-glucose pyrophosphorylase using protocol 2. Pooled aliquots from phenyl-Sepharose, MonoQ HR 5/5 and Superose 12 HR 10/30 columns containing ADP-glucose pyrophosphorylase activity were analysed on a 10-20% (w/v) SDS/polyacrylamide gel as described in the 'Materials and Methods' section. Samples contain approximately 25 μg protein per lane. Lane 1, low molecular weight marker proteins. Lane 2, crude extract of wheat endosperm (ADP-glucose pyrophosphorylase specific activity 9.38 nkat/mg protein). Lane 3, extract following chromatography on phenyl-Sepharose (ADP-glucose pyrophosphorylase specific activity 27.12 nkat/mg protein). Lane 4, extract after the first MonoQ HR 5/5 step (ADP-glucose pyrophosphorylase specific activity 150 nkat/mg protein). Lane 5, extract after the second MonoQ HR 5/5 step (ADP-glucose pyrophosphorylase specific activity 323 nkat/mg protein). Lane 6, extract following the Superose 12 HR 10/30 step (ADP-glucose pyrophosphorylase specific activity 691 nkat/mg protein). The arrow indicates the position of the 51 kD ADP-glucose pyrophosphorylase polypeptide. Molecular weights are indicated on the left.


M.W.
(x10⁻³)
1
2
3
4
5
6
94
67
43
30
20.1
14.4

the purification procedure were analysed on an SDS/polyacrylamide gel. Following gel filtration there were very few protein contaminants present in the enzyme preparation, which appeared to be greater than 90% pure in gels stained with PAGE blue 83 (Figure III.13). The results presented in Figure III.13 reveal the successive purification of a 51 kD polypeptide at each stage of the procedure. This polypeptide has the same molecular weight as the largest putative ADP-glucose pyrophosphorylase polypeptide purified using a different purification strategy (see Section III.2.A), suggesting that the polypeptide is indeed the ADP-glucose pyrophosphorylase subunit.

To prepare protein for N-terminal sequence analysis, 350 μg protein (of which at least 300 μg was estimated to be the 51 kD putative ADP-glucose pyrophosphorylase polypeptide) was fractionated on preparative SDS/polyacrylamide gels. The 51 kD protein band was excised and protein electroeluted as described in Section II.6.D. The protein sample was forwarded to I.C.I. Pharmaceuticals Division, Alderley Edge, Cheshire, U.K. for determination of the N-terminal sequence of the 51 kD polypeptide. No amino acid sequence was obtained for this protein.

C. Discussion

The purification of ADP-glucose pyrophosphorylase from wheat endosperm is reported here for the first time. In this report, the enzyme has been partially purified by two independent routes. The extent of purification reported for both procedures is similar to that reported for the maize endosperm ADP-glucose pyrophosphorylase enzyme (Plaxton and Preiss, 1987). The yields of wheat endosperm ADP-glucose pyrophosphorylase are routinely much lower than the 42% recovery reported for the maize enzyme (Plaxton and Preiss, 1987). This difference reflects the relative instability of the wheat endosperm ADP-glucose pyrophosphorylase enzyme. Gel filtration has not been employed previously in the purification of this enzyme from other sources; consequently, there are no data on whether the specific activity of other ADP-glucose pyrophosphorylases is reduced by protein concentration. In contrast to wheat endosperm ADP-glucose pyrophosphorylase, the enzymes from spinach leaf and potato tubers are quite stable. Heat-treatment at 60°C for 5 min and ammonium sulphate precipitation have been used in the purification of spinach leaf ADP-glucose pyrophosphorylase (Morell *et al*, 1987) and potato tuber ADP-glucose pyrophosphorylase (Sowokinos and Preiss, 1982), with recoveries of 71% and 83%, respectively. Both of these procedures inactivate the wheat endosperm enzyme (results not shown).

In the current study, both purification procedures which were employed yielded an active wheat enzyme with an apparent native molecular weight of 245,000 ± 30,000. This estimate is in agreement with the molecular weights for ADP-glucose pyrophosphorylases from several other sources, which range from 230,000 to 200,000; the maize endosperm enzyme is 230,000 (Plaxton and Preiss, 1987); the enzyme from *Rhodospirillum rubrum* is 225,000 (Furlong and Preiss, 1969); the *E.coli* enzyme is 210,000 (Haugen *et al*, 1974); the enzymes from both potato tuber (Sowokinos and Preiss, 1982) and spinach leaf (Morell *et al*, 1987) are 200,000.

The assignment of a 51 kD polypeptide for ADP-glucose pyrophosphorylase is indicated by the purification of this polypeptide from wheat endosperm using different purification procedures. Three major protein bands were resolved in the SDS/polyacrylamide gels of partially purified wheat endosperm ADP-glucose pyrophosphorylase, prepared using protocol 1 (Figure III.7). The possibilities that these three proteins are different molecular weight subunits of the same enzyme, or the subunits of different ADP-glucose pyrophosphorylase isoenzymes has been considered. However, one 51 kD polypeptide only was resolved in SDS/polyacrylamide gels of protein purified using protocol 2 (Figure III.13), indicating that the enzyme does not consist of different molecular weight subunits. In addition, no multiple peaks of enzyme activity were detected in the protein eluants from columns used in purification protocol 2, indicating that there was no selective purification of a wheat endosperm ADP-glucose pyrophosphorylase isoenzyme. Thus, the wheat endosperm ADP-glucose pyrophosphorylase enzyme subunits must have a molecular weight of 51,000. It is therefore likely that the smaller 50 kD and 49 kD polypeptides which were obtained using purification protocol 1, are proteolytic degradation products of the larger 51 kD polypeptide.

During the purification of enzyme activity according to purification protocol 1, proteolytic degradation of wheat endosperm ADP-glucose pyrophosphorylase may have occurred during overnight dialysis of the 39,000 x g supernatant; at this early stage of the purification the enzyme would be most likely to be in contact with contaminating proteases. As no proteolysis of ADP-glucose pyrophosphorylase was detectable when purification protocol 2 was employed, protease activity was probably removed from the endosperm extract during the initial purification step, i.e. chromatography on phenyl-Sepharose.

A subunit molecular weight of 51,000 for the wheat endosperm ADP-glucose pyrophosphorylase enzyme is similar to that reported for the enzymes from maize endosperm (M.W. 54,000; Plaxton and Preiss, 1987), *E.coli* (M.W. 51,000; Haugen *et al*, 1974) and potato tuber (M.W. 50,000; Sowokino and Preiss, 1982). Since the completion of this work, Krishnan *et al* (1986) have confirmed, by Western blotting of wheat grain extracts using anti-spinach leaf ADP-glucose pyrophosphorylase serum, that the wheat endosperm enzyme has a subunit molecular weight of the same approximate size (the estimated molecular weight of the polypeptide was reported as 50,000). Since the molecular weight of the native wheat endosperm enzyme is 245,000 ± 30,000 this suggests that the holoenzyme may be a tetrameric protein consisting of identical molecular weight subunits. On the basis of the current data, the possibilities that the wheat endosperm enzyme is pentameric or hexameric cannot be excluded. Determination of a protein's molecular weight by gel filtration is influenced by the shape of the molecule and is therefore subject to error. A true measure of the molecular weight of the purified native enzyme, determined by the sedimentation-equilibrium method is required.

ADP-glucose pyrophosphorylase purified according to purification protocol 2 was subjected to preparative SDS/polyacrylamide gel electrophoresis to obtain protein for N-terminal sequence analysis. These amino acid sequence data were to be used in the synthesis of mixed oligonucleotide probes to facilitate the screening of cDNA libraries. As mentioned in

Section III.2.B., no amino acid sequence was obtained from this protein sample. Since no sequence data were obtained during 8 cycles of degradation, it is concluded that the protein is blocked at the N-terminus. It is unlikely that insufficient protein was subjected to sequence analysis. Walker *et al* (1982) have reported the successful sequence analysis of only 100 pmol of protein. In the current experiment, the sample consisted of 6 nmol of ADP-glucose pyrophosphorylase protein. The protein was dialysed exhaustively following gel electrophoresis to remove Tris, glycine and SDS which might interfere with the sequence determination. Thus, unless these chemicals were complexed with the protein sample, contamination of the sample is unlikely to have been the reason for unsuccessful sequence analysis.

3. IMMUNOLOGICAL STUDIES OF WHEAT LEAF AND WHEAT ENDOSPERM ADP-GLUCOSE PYROPHOSPHORYLASE PROTEINS

A. Characterisation of anti-wheat endosperm ADP-glucose pyrophosphorylase sera

Antisera were prepared against the 51 kD ADP-glucose pyrophosphorylase polypeptide, as well as against the 50 kD and 49 kD proteolytic degradation products of this protein (see Section III.2.A,C). The immunisation of rabbits was carried out as described in Section II.8.B. Serum collected during the time-course of immunisation was assayed for the presence of specific antibodies as described in Section II.9.A. The results are presented in Figure III.14. Antibodies to the 51 kD polypeptide (immune serum I) and to the 50 kD polypeptide (immune serum II) were detectable 83 days following primary immunisation of rabbits. By 93 days after primary immunisation, 50% of the maximum binding of IgG to partially purified ADP-glucose pyrophosphorylase was observed in the solid phase ELISA with a 1/500 dilution of immune serum I, or a 1/1200 dilution of immune serum II (Figure III.14). In contrast, antibodies to the 49 kD polypeptide were not detected in any ELISA assays of blood serum.

Both immune sera I and II precipitated ADP-glucose pyrophosphorylase activity from crude extracts of wheat endosperm (Table III.3). Following incubation of extracts with immune serum I and centrifugation of the enzyme-IgG-protein A-Sepharose conjugates, only 10% of the ADP-glucose pyrophosphorylase activity remained in the supernatant fraction. Following incubation of extracts with immune serum II, only 3% of this enzyme's activity

Figure III.14: Serum antibody titres among animals immunised with putative ADP-glucose pyrophosphorylase proteins from wheat endosperm. Rabbits were immunised with either the 51 kD polypeptide (○), the 50 kD polypeptide (□), or the 49 kD polypeptide (◇), isolated from SDS/polyacrylamide gels as described in the 'Materials and Methods' section. The titres were determined by solid phase ELISA at the times after primary immunisation indicated on the abscissa. Serum dilutions that resulted in 50% of maximum binding of partially purified ADP-glucose pyrophosphorylase are indicated on the ordinate.

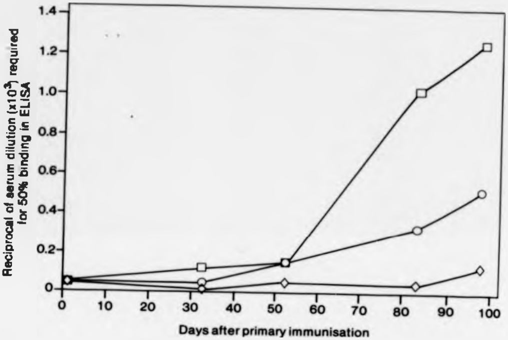

Reciprocal of serum dilution (x10^{3}) required for 50% binding in ELISA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
90
100
Days after primary immunisation

Table III.3 : Immunoprecipitation of ADP-glucose pyrophosphorylase activity by anti-wheat endosperm ADP-glucose pyrophosphorylase sera.

Incubation conditions	Enzyme activities (nkat/g fresh weight)[*]			
	ADP-glucose pyrophosphorylase		UDP-glucose pyrophosphorylase	Malate Dehydrogenase
	Supernatant	Pellet	Supernatant	Supernatant
Enzyme + preimmune serum I + protein A-Sepharose[1]	28.9 ± 2.4	0.54 ± 0.07	483.5 ± 14.1	2150.8 ± 104.7
Enzyme + immune serum I + protein A-Sepharose[1]	9.3 ± 1.3	21.24 ± 2.52	427.5 ± 15.5	1922.4 ± 137.6
Enzyme + preimmune serum II + protein A-Sepharose[2]	27.7 ± 1.1	0.36 ± 0.01	435.7 ± 5.3	1998.0 ± 61.1
Enzyme + immune serum II + protein A-Sepharose[2]	1.1 ± 0.1	21.42 ± 0.36	391.8 ± 26.3	1803.9 ± 118.8
Enzyme + protein A-Sepharose	30.5 ± 1.6	0.72 ± 0.18	445.8 ± 39.3	2084.6 ± 216.4

[*] Enzyme activity data is shown as the mean of three replicate samples ± S.E.M.

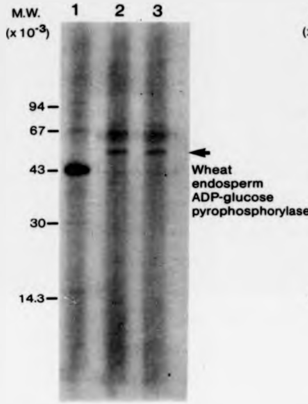
[1] Preimmune and immune serum I were obtained from a rabbit immunised with the 51 kD ADP-glucose pyrophosphorylase polypeptide.

[2] Preimmune and immune serum II were obtained from a rabbit immunised with the 50 kD ADP-glucose pyrophosphorylase polypeptide.

remained in the supernatant. The ADP-glucose pyrophosphorylase activity was detectable in the pellet fractions, in both circumstances, when the washed enzyme-Igγ-protein A-Sepharose conjugates were assayed directly for enzyme activity (Table III.3). Approximately 70% of the total enzyme activity present in the endosperm extracts was subsequently recovered as an immunoprecipitate in pellet fractions, following incubation with either immune serum. In control experiments, pre-immune sera collected from animals prior to their primary immunisation with ADP-glucose pyrophosphorylase did not partition ADP-glucose pyrophosphorylase activity. In addition, the incubation of wheat endosperm crude extracts with protein A-Sepharose alone did not result in the precipitation of enzyme activity. As shown in Table III.3, the activities of marker enzymes, UDP-glucose pyrophosphorylase and malate dehydrogenase, did not differ in supernatants of endosperm extracts treated with either protein A-Sepharose, pre-immune serum plus protein A—Sepharose, or anti-wheat endosperm ADP-glucose pyrophosphorylase sera plus protein A-Sepharose.

Western blot analysis of total wheat endosperm soluble protein using anti-wheat endosperm ADP-glucose pyrophosphorylase sera (i.e. immune serum I or immune serum II) revealed the presence of a 51 kD polypeptide in each case (Figure III.15). This finding supports the previous conclusion that the 50 kD and 49 kD polypeptides are proteolytic degradation products of the 51 kD ADP-glucose pyrophosphorylase polypeptide (see Section III.2.C). In a similar experiment, antibodies directed against the spinach leaf ADP-glucose pyrophosphorylase holoenzyme also recognised one polypeptide in wheat endosperm, of 51,000 molecular weight (Figure

Figure III.15: Western blot analysis of wheat endosperm proteins reacting with anti-wheat endosperm ADP-glucose pyrophosphorylase sera. Soluble protein was resolved on a 10-20% (w/v) SDS/polyacrylamide gel, transferred onto Hybond N membrane and probed with anti-wheat endosperm ADP-glucose pyrophosphorylase sera as described in the 'Materials and Methods' section. In panel A, the antiserum was prepared against the 51 kD ADP-glucose pyrophosphorylase polypeptide. In panel B, the antiserum used was prepared against the proteolysed 50 kD ADP-glucose pyrophosphorylase polypeptide. Lane 1 contains 3 μl of [^{14}C] methylated protein molecular weight markers. Lanes 2 and 3 contain 100 μg and 40 μg respectively, of a wheat endosperm soluble protein extract. Molecular weights are indicated on the left of each panel.


M.W.
(x 10⁻³)
1
2
3
94
67
43
30
14.3
Wheat endosperm ADP-glucose pyrophosphorylase

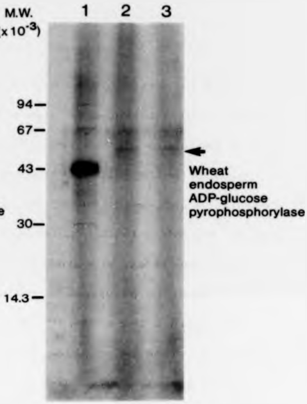
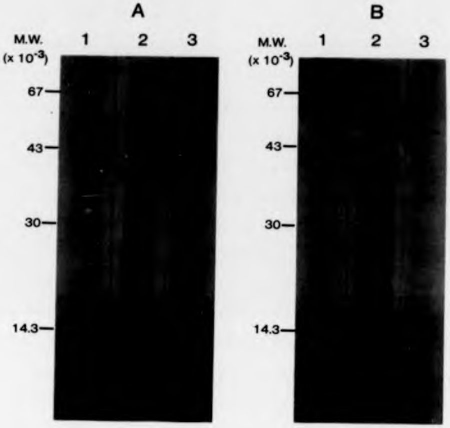

M.W.
(x 10⁻³)
1
2
3
94
67
43
30
14.3
Wheat endosperm ADP-glucose pyrophosphorylase

Figure III.16: Cross-reactivity between anti-wheat endosperm and anti-spinach leaf ADP-glucose pyrophosphorylase sera. Soluble protein (25 µg) was resolved on a 10-20% (w/v) SDS/polyacrylamide gel. Protein was either stained with PAGE blue 'Electran', or transferred onto Hybond N membrane and probed with antisera as described in the 'Materials and Methods' section. Lane 1 contains 3 µl of [^{14}C] methylated protein molecular weight markers. Lane 2 contains total wheat endosperm soluble protein stained with Page Blue 'Electran'. Lane 3 shows the signal obtained when Lane 2 proteins are blotted onto Hybond N membrane and then probed with antisera: In panel A, the blot was probed with anti-spinach leaf ADP-glucose pyrophosphorylase serum. In panel B, the blot was probed with anti-wheat endosperm ADP-glucose pyrophosphorylase serum. Molecular weights are indicated on the left of each panel.

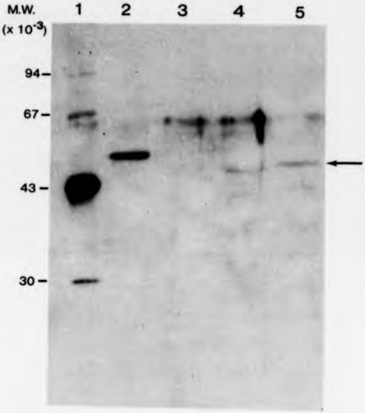

A
M.W.
(x 10⁻³)
1
2
3
67
43
30
14.3
B
M.W.
(x 10⁻³)
1
2
3
67
43
30
14.3

III.16, panel A, lane 3).

B. Subunit structures of wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase proteins

The presence of organ-specific isoenzymes of ADP-glucose pyrophosphorylase in wheat leaf and wheat endosperm was suggested by Western blot analysis of protein extracts from these organs, using anti-wheat endosperm ADP-glucose pyrophosphorylase serum to detect the enzyme subunits. As shown in Figure III.17, there was no immunoreactive protein band in the crude extracts of wheat leaves, although some background smudge around 60 kD was visible in both lane 3 and lane 4. The absence of an immunoreactive polypeptide in this case may have been due to the low titre of the antibodies employed. It also seemed possible that the abundance of the large subunit of ribulose bisphosphate carboxylase-oxygenase (Rubisco) in the region of 50,000 molecular weight obscured the ADP-glucose pyrophosphorylase polypeptide. Following chromatography of a wheat leaf crude extract on phenyl-Sepharose as described for the wheat endosperm enzyme (Section II.6.C), the protein extract was concentrated and loaded onto a column of Superose 12 HR 10/30 as described in Section II.6.B. The entire ultraviolet absorbing material which eluted from the gel filtration column after the void volume had passed through, was collected and analysed. This procedure was shown to reduce levels of Rubisco present in leaf extracts (result not shown). Following depletion of Rubisco from wheat leaf extracts, a single immunoreactive polypeptide with a molecular weight of

Figure III.17: Western blot analysis of wheat endosperm and wheat leaf ADP-glucose pyrophosphorylase polypeptides. soluble protein (50 μg) was resolved on a 10-20% (w/v) SDS/polyacrylamide gel, transferred onto Hybond N membrane and probed with anti-wheat endosperm ADP-glucose pyrophosphorylase serum as described in the 'Materials and Methods' section. Lane 1 contains 3 μl of [^{14}C] methylated protein molecular weight markers. Lane 2 contains wheat endosperm ADP-glucose pyrophosphorylase after phenyl-Sepharose chromatography (see Section III.2.C). Lane 3 contains a crude protein extract from wheat leaves. Lane 4 contains wheat leaf ADP-glucose pyrophosphorylase after chromatography on phenyl-Sepharose. Lane 5 contains wheat leaf ADP-glucose pyrophosphorylase after chromatography on phenyl-Sepharose and then Superose 12 HR 10/30. Molecular weights are indicated on the left. The position of the immunoreactive band in wheat leaf extracts is indicated


M.W.
(x 10^{-3})
1
2
3
4
5
94
67
43
30

Putative wheat
leaf ADP-glucose
pyrophosphorylase

48,500-49,000 was detected in Western blots (Figure III.17, lane 5). This protein is significantly smaller than the 51 kD wheat endosperm ADP-glucose pyrophosphorylase polypeptide (Figure III.17, lane 2).

C. Discussion

The purpose of this investigation was to characterise the wheat endosperm and wheat leaf ADP-glucose pyrophosphorylase enzymes on the basis of their subunit structures, thereby supplementingenzymological data which suggest the presence of organ-specific isoenzymes. To facilitate this analysis, anti-wheat endosperm ADP-glucose pyrophosphorylase sera have been prepared by the immunisation of rabbits with either the 51 kD ADP-glucose pyrophosphorylase polypeptide, or the 50 kD proteolytic digestion product of ADP-glucose pyrophosphorylase. This is the first report of the preparation of antibodies to the endosperm ADP-glucose pyrophosphorylase from a monocotyledonous plant species.

The specificities of the anti-wheat endosperm ADP-glucose pyrophosphorylase sera were indicated by immunoprecipitation and Western blotting experiments. Both immune sera were capable of specifically immunoprecipitating at least 70% of the ADP-glucose pyrophosphorylase activity from crude extracts of wheat endosperm (Table III.3).

In Western blot experiments with wheat endosperm soluble protein, a single antigenic polypeptide was detected, with an apparent molecular weight of 51,000 (Figure III.15). This polypeptide was recognised by antibodies prepared against either the 51 kD wheat endosperm ADP-glucose pyrophosphorylase polypeptide or the 50 kD proteolysed wheat endosperm ADP-glucose pyrophosphorylase polypeptide. In addition, antibodies prepared against the native spinach leaf ADP-glucose pyrophosphorylase enzyme also recognise this 51 kD polypeptide in Western blotting experiments (Figure III.16), suggesting that there are some similarities in the primary structures of the spinach leaf and wheat endosperm enzymes. Since the completion of these experiments, cross-reactivity of the spinach leaf ADP-glucose pyrophosphorylase with the rice endosperm, rice leaf, maize endosperm and maize leaf ADP-glucose pyrophosphorylase subunits has also been reported (Krishnan *et al*, 1986). The observations of Krishnan *et al* (1986) indicate that ADP-glucose pyrophosphorylase is highly conserved within the plant kingdom. J. Preiss (Dept of Biochemistry, Michigan State University, East Lansing, Michigan, U.S.A.; personal communications) has also observed immunological cross-reactivity of the enzymes from spinach leaf and *E.coli*, and so it is possible that there are some highly conserved regions of the protein between *E.coli* and the higher plants. In support of this suggestion, a comparison of the protein sequences of *E.coli* ADP-glucose pyrophosphorylase and wheat ADP-glucose pyrophosphorylases is presented in Section III.5.F. and reveals 24% identity overall. In addition, there is as much as 50% identity between wheat and *E.coli* polypeptides in certain regions, for example between residues 62-117 and residues 151-173 of the wheat endosperm ADP-glucose pyrophosphorylase sequence.

The presence of organ-specific isoenzymes of ADP-glucose pyrophosphorylase in the leaves and endosperm of wheat is suggested by the results of Western blot experiments conducted in the current study. In Western blots of Rubisco-depleted wheat leaf extracts probed with anti-wheat endosperm ADP-glucose pyrophosphorylase serum, a single polypeptide was detected with an apparent molecular weight of 48,500-49,000 (Figure III.17). The wheat leaf ADP-glucose pyrophosphorylase polypeptide is therefore approximately 2.5 kD smaller than the corresponding polypeptide from wheat endosperm. From these data alone however, one cannot exclude the possibility that the 48.5 kD leaf polypeptide is a processed form of the 51 kD endosperm polypeptide. The identification and determination of nucleotide sequences for cDNAs encoding wheat endosperm and wheat leaf ADP-glucose pyrophosphorylase polypeptides, will be necessary to resolve these possibilities.

Since the completion of this work two years ago, the presence of organ-specific isoenzymes of wheat ADP-glucose pyrophosphorylase has also been suggested by Krishnan *et al* (1986). The molecular weight estimate obtained by Krishnan *et al* (1986) for the wheat endosperm enzyme subunit is 50,000, consistent with the current findings. However, these authors have detected two polypeptides of wheat leaf ADP-glucose pyrophosphorylase, of 46,000 and 43,000 apparent molecular weight, in crude extracts of wheat leaves. This result differs significantly from the current findings presented in this thesis. It is my experience that the abundance of Rubisco in protein extracts produces distorted migration of proteins with similar sizes to the Rubisco subunits in SDS/polyacrylamide gels. As a consequence, the molecular weight estimates obtained by Krishnan *et al* (1986) for the wheat leaf ADP-glucose pyrophosphorylase subunits may be incorrect. It is also noteworthy that the anti-spinach leaf ADP-glucose pyrophosphorylase serum used in those experiments has since been found to contain antibodies against Rubisco enzyme (Morell *et al*, 1987). It is therefore possible that the 46 kD polypeptide observed by Krishnan *et al* (1986) was the large subunit of Rubisco. An alternative explanation consistent with the observations of both the current study and those of Krishnan *et al* (1986), is that the wheat leaf enzyme consists of two subunits, of different molecular weight, and only one of these subunits cross-reacts immunologically with the wheat endosperm subunit. At present it is not possible to discount this explanation and further experiments on the subunit structure of wheat leaf ADP-glucose pyrophosphorylase are required to clarify the situation. If this alternative explanation is confirmed, it may have important implications in terms of the allosteric regulation of ADP-glucose pyrophosphorylase. One may speculate that the allosteric properties of the wheat leaf enzyme are conferred by the leaf-specific subunit (i.e the subunit which does not cross-react immunologically with wheat endosperm ADP-glucose pyrophosphorylase). Since this subunit is absent from wheat endosperm ADP-glucose pyrophosphorylase, the lack of allosteric activation of the endosperm enzyme by 3-phosphoglycerate would be explained.

The same controversy currently exists over the subunit structure of the spinach leaf ADP-glucose pyrophosphorylase. Denatured preparations of purified spinach leaf ADP-glucose pyrophosphorylase enzyme contain two polypeptides of 54,000 and 51,000 molecular weight which do not cross-react immunologically and have different tryptic digest patterns and different N-terminal amino acid sequences (Morell *et al*, 1987). Antibodies prepared against the 51 kD polypeptide of the spinach enzyme cross-react with the 54 kD subunit of purified maize endosperm ADP-glucose pyrophosphorylase, while antibodies prepared against the larger 54 kD spinach leaf ADP-glucose polypeptide do not cross-react with the maize endosperm protein (Morell *et al*, 1987). However, these authors concede that the larger spinach polypeptide may be a contaminant in the ADP-glucose pyrophosphorylase preparations. There is currently no technique available for definitely assigning ADP-glucose pyrophosphorylase activity to a particular polypeptide visualised in an SDS/polyacrylamide gel. Thus, all that can be done is to purify the enzyme activity to the highest extent possible.

The suggestion that wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase proteins may be the product of different genes, is consistent with the observation of Fentem and Catt (I.C.I. Plant Biotechnology Group, Runcorn, Cheshire, U.K.; personal communications) that the ADP-glucose pyrophosphorylase activity in these organs is differentially activated by 3-phosphoglycerate and inhibited by orthophosphate. A comparison of the nucleotide and deduced amino acid sequences of cDNAs encoding wheat leaf and wheat endosperm ADP-glucose pyrophosphorylases will help us to understand more completely the different allosteric properties of these organ-specific isoenzymes.

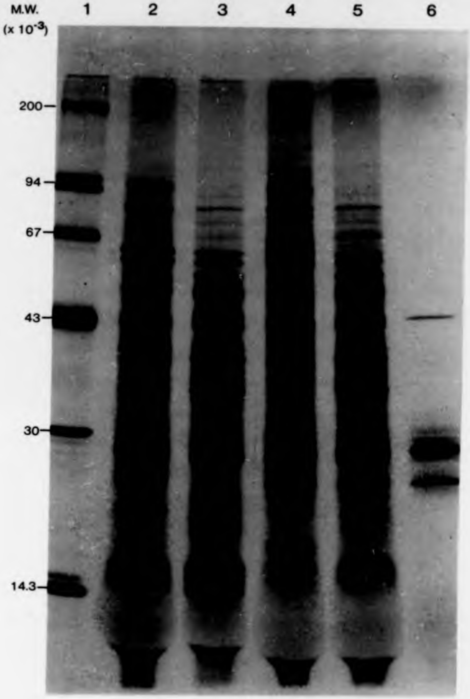
4. MESSENGER RNA STUDIES

A. Isolation and *in vitro* translation of RNA from wheat endosperm

In routine preparations of free polysomes (FP) and membrane-bound polysomes (MBP) from 15-day old wheat endosperms, the yield of RNA from each polysome fraction was approximately 200 µg/g fresh weight. After the purification of poly(A)-containing RNA from polysomal RNA samples, approximately 2% (w/w) of FP RNA and 4.2% (w/w) of MBP RNA was recovered in the poly(A)-containing fraction.

Polysomal RNA and poly(A)-containing RNA samples, extracted from FP and MBP fractions, were translated *in vitro* in a rabbit reticulocyte cell-free translation system as described in Section II.13. The optimal conditions for *in vitro* translation of these RNA samples were determined in preliminary experiments. L-[^{35}S]methionine was routinely incorporated into protein to an approximate specific activity of 4x10^{5} c.p.m./µg RNA for poly(A)-containing RNA and 5x10^{3} c.p.m./µg RNA for total polysomal RNA. The translation products of FP and MBP RNA samples were subsequently analysed on a 10-20% (w/v) SDS/polyacrylamide gel; the results are presented in Figure III.18. Under the assay conditions described in Section II.13, FP RNA and MBP RNA were able to direct the synthesis of a large number of polypeptides, with apparent molecular weights ranging from 20,000 to 95,000 in the case of FP RNA, and from 20,000 to 130,000 in the case of MBP RNA. The majority of polypeptides had molecular weights greater than 30,000, indicating that RNase activity was probably not a problem encountered in the purification and handling of RNA

Figure III.18: The *in vitro* translation products of wheat endosperm polysomal RNAs. RNA was extracted from the free polysomes and membrane-bound polysomes of 15-day old wheat endosperms and translated *in vitro* in the presence of L-[^{35}S] methionine, as described in the 'Materials and Methods' section. The translation products were analysed on a 10-20% (w/v) SDS/polyacrylamide gel. Lane 1 contains 3 μl of [^{14}C] methylated protein molecular weight markers. Lane 2 contains the translation products of total free polysomal RNA. Lane 3 contains the translation products of total membrane-bound polysomal RNA. Lane 4 contains the translation products of poly(A)-containing free polysomal RNA. Lane 5 contains the translation products of poly(A)-containing membrane-bound polysomal RNA. Lane 6 contains endogenous translation products (without added RNA). Molecular weights are indicated on the left.


M.W.
(x 10⁻³)
1
2
3
4
5
6
200
94
67
43
30
14.3

samples.

As shown in Figure III.18, the polypeptide profiles of FP RNA and MBP RNA translation products differ significantly (compare lanes 2 and 3), indicating that there may have been little contamination of FP RNA with disrupted membrane-bound polysomes in these preparations. Two highly abundant polypeptides with apparent molecular weights of 60,000 and 63,000 were present in the polypeptide profiles obtained by *in vitro* translation of FP RNA (Figure III.18, lane 2). The identity of these polypeptides is not known, but they may be amyloplast or mitochondrial proteins, since the abundance of both polypeptides is reduced in the translation products of FP-poly(A)-containing RNA samples (Figure III.18, lane 4). The polypeptide profiles of total FP RNA and FP-poly(A)-containing RNA are similar in other respects.

The MBP RNA translation products are characterised by at least three size classes of polypeptide, with 42,000-46,000, 32,000-40,000 and 13,000-20,000 apparent molecular weights. On the basis of their apparent molecular weights, polypeptides in the 42,000-46,000 and the 32,000-40,000 size classes are probably α-,β- and γ-gliadins; these polypeptides have been previously characterised and are synthesised exclusively on membrane-bound polysomes (Greene, 1981; Donovan *et al*, 1982). Polypeptides belonging to the 13-20 kD size class have been observed by Okita and Greene (1982) in the translation products of 20-day old wheat endosperm, but their identity is not known. The translation products of MBP RNA also contain a major 55 kD polypeptide (Figure III.18), which may be ω-gliadin (Forde and Miflin, 1983). The high-molecular weight prolamin polypeptides are also present in the translation products of MBP RNA, in the region of 80-130 kD

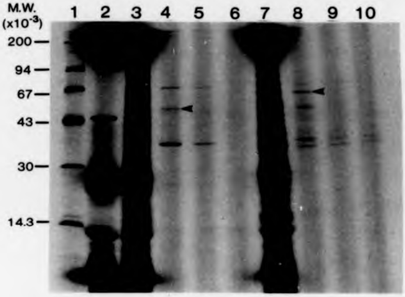
(Figure III.18; Forde and Miflin, 1983). The translation of MBP-poly(A)-containing RNA produced a similar polypeptide profile to that obtained by *in vitro* translation of total MBP RNA (Figure III.18, compare lanes 3 and 5), suggesting that most MBP RNA species are polyadenylated.

B. Identification of the *in vitro* translation products of wheat ADP-glucose pyrophosphorylase mRNAs

Poly(A)-containing RNAs from FP and MBP fractions of wheat endosperm were translated *in vitro*, and the resulting ADP-glucose pyrophosphorylase translation products were immunoprecipitated with anti-wheat endosperm ADP-glucose pyrophosphorylase serum. The results are presented in Figure III.19. Interestingly, a polypeptide with an apparent molecular weight of 61,000 was immunoprecipitated from the translation products of MBP-poly(A)-containing RNA (Figure III.19, lane 8), while a polypeptide of approximately 53,000 molecular weight was immunoprecipitated from the translation products of FP-poly(A)-containing RNA (Figure III.19, lane 4). There was also a trace of the 53 kD polypeptide in the immunoprecipitate of the translation products of MB-poly(A)-containing RNA. As shown in Figure III.19, neither of these polypeptides was precipitated by incubation of samples with pre-immune serum, or protein A-Sepharose alone. In contrast, polypeptides of 67,000, 35,000 and 33,000 molecular weight were non-specifically precipitated by pre-immune serum and immune serum.

When poly(A)-containing RNA isolated from the free polysomes of

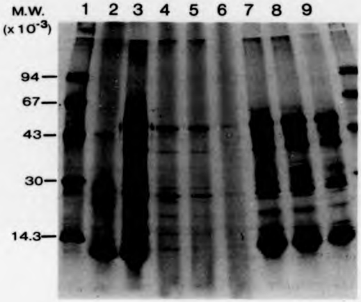
Figure III.19: Identification of the *in vitro* translation products of wheat endosperm ADP-glucose pyrophosphorylase mRNA. Poly(A)-containing RNA extracted from free polysomes or membrane-bound polysomes of wheat endosperm was translated *in vitro*. Specific translation products were subsequently immunoprecipitated by incubation with anti-wheat endosperm ADP-glucose pyrophosphorylase serum as described in the 'Materials and Methods' section. The immunoprecipitates were analysed on a 10-20% (w/v) SDS/polyacrylamide gel. Lane 1 contains 3 μl of [^{14}C] methylated protein molecular weight markers. Lane 2 contains endogenous translation products (without added RNA). Lane 3 contains the translation products of RNA extracted from free polysomes. Lane 4 contains the immunoprecipitate from Lane 3 following incubation with antiserum. Lane 5 contains the immunoprecipitate from Lane 3 following incubation with pre-immune serum. Lane 6 contains the immunoprecipitate from Lane 3 following incubation with protein A-Sepharose. Lane 7 contains the translation products of RNA extracted from membrane-bound polysomes. Lane 8 contains the immunoprecipitate from Lane 7 following incubation with antiserum. Lane 9 contains the immunoprecipitate from Lane 7 following incubation with pre-immune serum. Lane 10 contains the immunoprecipitate from Lane 7 following incubation with protein A-Sepharose. Arrows indicate the positions of putative ADP-glucose pyrophosphorylase polypeptides. Molecular weights are indicated on the left.


M.W.
(x10⁻³)
200
94
67
43
30
14.3
1
2
3
4
5
6
7
8
9
10

wheat leaves was translated *in vitro* and the translation products analysed by SDS/polyacrylamide gel electrophoresis, the polypeptide profile shown in Figure III.20 was produced. In the translation of wheat leaf RNA, major polypeptides were synthesised with apparent molecular weights of 50,000, 38,000 and 20,000 (Figure III.20). On the basis of their molecular weights, these polypeptides were tentatively identified as the large subunit of Rubisco (M.W. 50,000), and the precursors of the light harvesting chlorophyll a/b protein (M.W. 38,000) and the small subunit of Rubisco (M.W. 20,000). In order to visualise additional, less abundant polypeptides in these translation products, long (1 month) exposures of X-ray film to the gel were required. Identical polypeptide profiles have been obtained for the *in vitro* translation products of total RNA from wheat leaves, when translated in the rabbit reticulocyte lysate (result not shown).

Following incubation of the translation products of wheat leaf poly(A)- containing RNA with anti-wheat endosperm ADP-glucose pyrophosphorylase serum, no specific immunoprecipitates were detected (Figure III.20, compare lanes 4 and 5). Similar results were obtained when anti-spinach leaf ADP-glucose pyrophosphorylase serum was used in the immunoprecipitation experiment. In several attempts to obtain a result with this experiment, there was always a high degree of non-specific precipitation of the abundant polypeptides. The inability to obtain an imunoprecipitate of the wheat leaf ADP-glucose pyrophosphorylase may suggest that the mRNA encoding wheat leaf ADP-glucose pyrophosphorylase is not be very abundant relative to other leaf mRNA species. However, the possibility that the translation of ADP-glucose pyrophosphorylase mRNA was inhibited by some contaminant in the RNA samples cannot

Figure III.20: Attempted immunoprecipitation of the wheat leaf ADP-glucose pyrophosphorylase primary translation product. Wheat leaf poly(A)-containing RNA extracted from wheat polysomes was translated *in vitro* in the presence of L-[^{35}S] methionine, in a rabbit reticulocyte cell-free translation system. The translation products were incubated with anti-wheat endosperm ADP-glucose pyrophosphorylase serum in the presence or absence of purified wheat endosperm ADP-glucose pyrophosphorylase as described in the 'Materials and Methods' section. The immunoprecipitates were subsequently analysed on a 10-20% (w/v) SDS/polyacrylamide gel. Lane 1 contains 3 µl of [^{14}C] methylated protein molecular weight markers. Lane 2 contains endogenous translation products (without added RNA). Lane 3 contains the translation products of wheat leaf poly(A)-containing RNA. Lane 4 contains the immunoprecipitate from Lane 3 products incubated with antiserum. Lane 5 contains the immunoprecipitate from Lane 3 following incubation with antiserum plus 20 µg of purified wheat endosperm ADP-glucose pyrophosphorylase. Lane 6 contains the immunoprecipitate from Lane 3 following incubation with protein A-Sepharose (i.e. no antiserum). Lane 7 is the supernatant obtained following centrifugation to remove the immunoprecipitates shown in Lane 4. Lane 8 is the supernatant obtained following centrifugation to remove the immunoprecipitates shown in Lane 5. Lane 9 is the supernatant obtained following centrifugation to remove the immunoprecipitates shown in Lane 6. Molecular weights are indicated on the left.


M.W.
(x 10^{-3})
1
2
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94
67
43
30
14.3

be discounted. It is unlikely that the RNA sample was degraded by nucleases, since the same RNA was later shown to produce reasonable hybridisation signals in Northern blotting experiments (see Section III.5.A).

C. Discussion

The purpose of these experiments was to identify the primary translation products of wheat endosperm and wheat leaf ADP-glucose pyrophosphorylase mRNAs, by immunoprecipitation of the nascent polypeptides. Two polypeptides have been immunoprecipitated from the translation products of wheat endosperm ADP-glucose pyrophosphorylase mRNA, with apparent molecular weights of 61,000 and 53,000. The estimated molecular weights for these polypeptides are in close agreement with those obtained more recently by Krishnan *et al* (1986). These authors have immunoprecipitated polypeptides from wheat grain total poly(A)-containing RNA, with apparent molecular weights of 56,000 and 50,000. Krishnan *et al* (1986) have concluded that the smaller polypeptide is a proteolytic digestion product of the 56 kD polypeptide. In the present study, the 61 kD putative ADP-glucose pyrophosphorylase precursor polypeptide is synthesised exclusively on membrane-bound polysomes, while the smaller 53 kD polypeptide has been found associated primarily with free polysomes. If the 53 kD polypeptide were a proteolytic digestion product of the larger 61 kD polypeptide, we would expect to find the same proportion of 61 kD polypeptide to 53 kD polypeptide in the translation products of FP RNA and MBP RNA. Since this is clearly not the case, the 61 kD and

53 kD polypeptides are probably the products of different genes.

Despite the observed fractionation of putative precusor ADP-glucose pyrophosphorylase polypeptides obtained in these experiments, the *in vivo* site of synthesis of the 53 kD polypeptide is not certain. This is because of the presence of trace amounts of the 53 kD ADP-glucose pyrophosphorylase polypeptide in the translation products of MBP-poly(A)-containing RNA. It is possible that these polysomes have become stripped from the endoplasmic reticulum during the isolation procedure. Consequently, the possibility that the 53 kD ADP-glucose pyrophosphorylase polypeptide is synthesised *in vivo* on membrane-bound polysomes cannot be discounted, although this would be inconsistent with what is already known about the site of synthesis of nuclear-encoded chloroplast and mitochondrial proteins (see below; Schmidt and Mishkind, 1986).

The 61 kD putative ADP-glucose pyrophosphorylase precursor polypeptide is almost certainly synthesised *in vivo* on membrane-bound polysomes, since there were no traces of this protein in the translation products of FP-poly(A)-containing RNA, as might be expected if free polysomes were trapped in the membrane pellet during the fractionation procedure. The association of ADP-glucose pyrophosphorylase mRNA with membrane-bound polysomes is surprising, since there are no other examples of nuclear-encoded organelle proteins synthesised on the rough endoplasmic reticulum. In addition, there is no known route from the lumen of the endosplasmic reticulum to the plastid. Generally, only secretory proteins are known to be synthesised on membrane-bound polysomes, and these polypeptides are transported from the lumen of the endoplasmic reticulum to their final destination via the golgi apparatus. Since wheat endosperm ADP-glucose pyrophosphorylase is an amyloplast protein, it is possible that transport of polypeptides to this organelle occurs by a different mechanism to that which has been characterised for chloroplasts and mitochondria. The possibility that the antibody used in the immunoprecipitation experiment was not mono-specific has also been considered. This antibody reacts immunologically with a single 51 kD polypeptide in Western blots (see Section III.3.A) and is therefore mono-specific by this criterion. However, if the immune serum contained antibodies to polypeptides of a similar molecular weight to the mature ADP-glucose pyrophosphorylase subunit, this would not be detected in Western blot experiments. If so, both the 61 kD polypeptide and the 53 kD polypeptide may not be ADP-glucose pyrophosphorylase precursors. To determine whether this latter explanation is correct, it will be necessary to repeat this experiment using different ADP-glucose pyrophosphorylase antisera. The identity of the 53 kD precursor ADP-glucose pyrophosphorylase polypeptide has been confirmed by immunoprecipitation of the *in vitro* translatior products of FP poly(A)-containing RNA using anti-spinach leaf ADP-glucose pyrophosphorylase serum. In this experiment, purified wheat endosperm ADP-glucose pyrophosphorylase successfully competed with the nascent 53 kD polypeptide for antibody binding (results not shown). Unfortunately, there was insufficient anti-spinach leaf ADP-glucose pyrophosphorylase serum at my disposal to confirm the identity of the 61 kD polypeptide.

The mature wheat endosperm ADP-glucose pyrophosphorylase polypeptide has a molecular weight of approximately 51,000 (see Section III.2., Section III.3). The 53 kD *in vitro* translation product of wheat endosperm ADP-glucose pyrophosphorylase mRNA has a molecular weight very similar to the mature protein, indicating that it probably has a small N-terminal extension, which is involved in targeting of the protein to the amyloplast. Since the 61 kD polypeptide is much larger than the mature ADP-glucose pyrophosphorylase polypeptide, it probably contains a presequence at the N-terminus of approximately 10,000 molecular weight, assuming that it is indeed an ADP-glucose pyrophosphorylase precursor. Nucleotide sequence analysis of a cDNA encoding wheat endosperm ADP-glucose pyrophosphorylase (clone WE:AGA.7) reveals the presence of a sequence at the N-terminus of the derived polypeptide which contains a similar amino acid composition to the transit peptide of chloroplast proteins (see Section III.5.G.2). If this polypeptide is synthesised on membrane-bound polysomes and transported to the amyloplast via golgi vesicles, then the question arises as to the functional requirement for this transit sequence, since the polypeptide itself may not need to cross the amyloplast membrane.

The sizes of the nascent wheat endosperm ADP-glucose pyrophosphorylase polypeptides indicate that the mRNAs encoding them are of at least 1400 bp in length in the case of the 53 kD polypeptide, and 1700 bp in the case of the 61 kD polypeptide. This estimate assumes that an amino acid in a polypeptide has an average molecular weight of 110 and does not allow for the presence of 5'-untranslated and 3'-untranslated sequences in the mRNAs. This approximation is in close agreement with the size of wheat endosperm ADP-glucose pyrophosphorylase mRNA estimated in Northern blotting experiments to be approximately 1.8 kb (see Section III.5.A).

In these experiments, the immunoprecipitation of a primary translation product of wheat leaf ADP-glucose pyrophosphorylase mRNA has not been achieved. The possible reasons for this have been mentioned in Section III.4.B. Krishnan *et al* (1986) have reported the immunoprecipitation of two ADP-glucose pyrophosphorylase polypeptides from the translation products of wheat leaf poly(A)-containing RNA, with apparent molecular weights of 76,000 and 73,000. The estimated molecular weights of the presequences of these polypeptides are 27,000 and 24,000, based on a molecular weight of 48,500-49,000 for the mature wheat leaf protein (see Section III.3.B.). These presequences are much larger than any other characterised presequence for a chloroplast protein, which is usually of 4-6 kD (Schmidt and Mishkind, 1986). If the reported sizes of the nascent wheat leaf ADP-glucose pyrophosphorylase polypeptides are correct, then the coding information alone would require 2.1 kb of mRNA, assuming that an amino acid has an average molecular weight of 110. This estimate does not allow for the presence of 5'-untranslated and 3'-untranslated sequences in the mRNA and yet it is excess of the 1.7 kb size estimate for wheat leaf ADP-glucose pyrophosphorylase mRNA, obtained in Northern blot experiments (see Section III.5.A.). Further experiments are required to confirm the observations of Krishnan *et al* (1986), on the identification of a primary translation product of wheat leaf ADP-glucose pyrophosphorylase mRNA.

5. ISOLATION AND CHARACTERISATION OF WHEAT ADP-GLUCOSE PYROPHOSPHORYLASE cDNAs

A. Isolation of a cDNA encoding wheat leaf ADP-glucose pyrophosphorylase

A wheat leaf cDNA library of $1.2\text{x}10^6$ independent cDNA sequences in the λgt11 expression vector was screened with anti-wheat endosperm ADP-glucose pyrophosphorylase serum as described in Section II.16.A. The library consisted of 85-90% recombinant phage and had been previously amplified in *E.coli* strain Y1088, according to Huynh *et al* (1985). The final titre of bacteriophage in the amplified cDNA library was $3\text{x}10^{10}$ p.f.u./ml (C. Raines, Plant Breeding Institute, Trumpington, Cambridge, U.K.; personal communications). In an initial screen of $1\text{x}10^5$ bacteriophage particles, sixty clones producing signals were detected. Ten of these clones which produced the strongest signals were further screened for the production of β-galactosidase/ ADP-glucose pyrophosphorylase fusion proteins. Of these ten clones, four screened positive in successive rounds of screening.

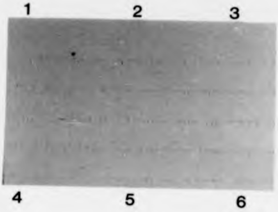
DNA was prepared from the putative wheat leaf ADP-glucose pyrophosphorylase cDNA clones as described in Section II.17. Following endonuclease restriction of bacteriophage DNA with *Eco*R1 and agarose gel electrophoresis, the cDNA inserts were shown to range in size from 600 bp to 1450 bp. However, in hybridisations of Southern blots to the cDNA inserts, none of these putative ADP-glucose pyrophosphorylase cDNAs were shown to cross-hybridise, indicating that none of the clones contained related cDNA sequences (result not shown). Thus, it was apparent that not all of the clones encoded ADP-glucose pyrophosphorylase. As shown in Figure III.21A, a weak signal is produced on screening these cDNA clones with anti-wheat endosperm ADP-glucose pyrophosphorylase serum, relative to the signal produced by non-recombinant λgt11. It is believed that this weak signal is due to the low titre of antibodies in that immune serum. Unfortunately, when the dilution of anti-wheat endosperm ADP-glucose pyrophosphorylase serum used in the first round of immunological screening was reduced to 1/50, at the same concentration of second antibody, then every plaque on the plate produced a signal. Similarly, when the antibody was used at dilutions greater than 1/100, then the signals produced were even weaker than those presented in Figure III.21A (results not shown).

The four putative ADP-glucose pyrophosphorylase cDNA clones were subsequently screened with the high-titre anti-spinach leaf ADP-glucose pyrophosphorylase serum. Since previous experiments indicate that this antibody is able to detect wheat endosperm ADP-glucose pyrophosphorylase in Western blots (see Section III.3.A.), it seemed reasonable to assume that it should also be able to detect epitopes of the wheat leaf ADP-glucose pyrophosphorylase protein. The results of this screen (Figure III.21B) indicate that none of these cDNA clones produce fusion proteins recognised by anti-spinach leaf ADP-glucose pyrophosphorylase serum. Therefore, it was concluded that none of the cDNA clones purified using anti-wheat endosperm ADP-glucose pyrophosphorylase serum, were true wheat leaf ADP-glucose pyrophosphorylase cDNA clones.

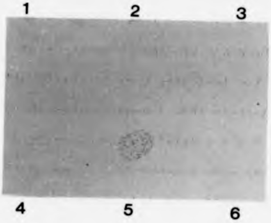
In a second attempt to identify a cDNA clone encoding wheat

Figure III.21: Immunological screen of putative wheat leaf ADP-glucose pyrophosphorylase cDNA clones. The recombinant bacteriophage were plaque-purified using anti-wheat endosperm ADP-glucose pyrophosphorylase or anti-spinach leaf ADP-glucose pyrophosphorylase serum as described in the 'Materials and Methods' section. Freshly plated lawns of *E.coli* strain Y1088 were infected with 5 µl of each bacteriophage containing 100 p.f.u. and immunological screening carried out as previously indicated. Figure A shows the results obtained from screening plaques with anti-wheat endosperm ADP-glucose pyrophosphorylase serum. Figure B shows the results obtained from screening plaques with anti-spinach leaf ADP-glucose pyrophosphorylase serum. Bacteriophage 1-4 inclusive were initially plaque-purified using anti-wheat endosperm ADP-glucose pyrophosphorylase serum. Bacteriophage 5 was initially plaque-purified using anti-spinach leaf ADP-glucose pyrophosphorylase serum. Bacteriophage 6 is a non-recombinant λgt11 included as a negative control.

A



B



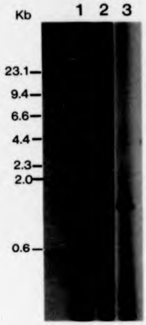
leaf ADP-glucose pyrophosphorylase, 3×10^4 bacteriophage from the amplified wheat leaf cDNA library were screened with anti-spinach leaf ADP-glucose pyrophosphorylase serum as described in Section II.16.A. Of seven positive clones selected on the first screening, three clones producing the strongest signals were re-screened. Only one of these clones produced strong positive signals in subsequent rounds of screening. The intensity of the signal produced by this cDNA clone, relative to non-recombinant λgt11 (see Figure III.21.B) suggests that the clone contains a cDNA encoding wheat leaf ADP-glucose pyrophosphorylase. The fusion protein produced by this clone is not recognised by anti-wheat endosperm ADP-glucose pyrophosphorylase serum (Figure III.21A), which is probably a reflection on the low antibody titre of that immune serum. The putative wheat leaf ADP-glucose pyrophosphorylase cDNA clone was designated WL:AGA.1. At this time a decision was taken to characterise clone WL:AGA.1 more completely. As a consequence of that decision, the remaining four positive signals were not investigated further.

Bacteriophage DNA was prepared from clone WL:AGA.1, as described in Section II.17.A. Following restriction endonuclease digestion of the DNA with the enzyme *Eco*R1, clone WL:AGA.1 was shown to contain a cDNA insert of 950 bp in size (Table III.4). The cDNA insert was labelled with ^{32}P by nick-translation and used to probe a Northern blot of poly(A)-containing RNA from wheat leaf and wheat endosperm (Figure III.22). This probe hybridises to mRNA bands of approximately 1.7 kb and 1.8 kb in size, respectively, in the leaf and endosperm RNA samples. The sizes of the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase mRNAs determined here are in close agreement with the estimates obtained by

Table III.4 : Summary of the sizes of wheat ADP-glucose pyrophosphorylase cDNA inserts

Organ in which cDNA expressed	cDNA clone	Approximate size of insert (bp)
Leaf	WL : AGA.1	950
Endosperm	WE : AGA.1	1600
Endosperm	WE : AGA.3	1250
Endosperm	WE : AGA.4	400
Endosperm	WE : AGA.5	1200
Endosperm	WE : AGA.6	1600
Endosperm	WE : AGA.7	1800

Figure III.22: Northern blot of wheat endosperm and wheat leaf poly(A)-containing RNA probed with cDNA clone WL:AGA.1. Poly(A)-containing RNA (1 μg), or purified cDNA insert from the wheat leaf ADP-glucose pyrophosphorylase cDNA clone WL:AGA.1, was glyoxalated and resolved on a 1% (w/v) agarose gel. Glyoxalated *Hin*dIII fragments of bacteriophage lambda DNA were used as size markers. The nucleic acids were blotted onto GeneScreen Plus membrane and hybridised to the nick-translated cDNA insert of clone WL:AGA.1 as described in the 'Materials and Methods' section. Lane 1 contained wheat endosperm poly(A)-containing RNA. Lane 2 contained wheat leaf poly(A)-containing RNA. Lane 3 contained the purified cDNA insert of clone WL:AGA.1. Size marker fragment lengths are indicated on the left.


Kb
1
2
3
23.1
9.4
6.6
4.4
2.3
2.0
0.6

Krishnan *et al* (1986), from Northern blots of wheat RNAs probed with a cDNA encoding rice endosperm ADP-glucose pyrophosphorylase. Moreover, there would be sufficient capacity in the 1.8 kb mRNA sequence to code for a polypeptide of 61 kD, the size of the larger putative wheat endosperm ADP-glucose pyrophosphorylase precursor (see Section III.4.B.). Comparison of the size of the cDNA insert with the size of the homologous mRNA species, indicates that the WL:AGA.1 cDNA insert contains approximately 55% of the complete wheat leaf ADP-glucose pyrophosphorylase mRNA sequence. Unfortunately, there was insufficient time during the course of this Ph.D. programme to re-screen the wheat leaf cDNA library with a view to isolating a full-length wheat leaf ADP-glucose pyrophosphorylase cDNA clone.

B. Isolation of cDNAs encoding wheat endosperm ADP-glucose pyrophosphorylase

A wheat endosperm cDNA library was constructed as described in Section II.14. Double-stranded cDNA was prepared from oligo dT-cellulose-purified wheat endosperm RNA by a method employing RNaseH and *E.coli* DNA polymerase I in the synthesis of the second strand, without prior purification of single-stranded cDNA (Gubler and Hoffman, 1983). This method does not produce terminal hairpin loops, as do other methods which remove the mRNA after the first strand of cDNA is synthesised. Consequently, there is no need in the method of Gubler and Hoffman (1983) to remove terminal hairpin bends from the double-stranded cDNA by S_1-nuclease digestion. Thus, there is a greater probability that cDNAs produced using the

RNaseH method will be full-length. From 2.4 μg of poly(A)-containing wheat endosperm RNA, 450 ng of single-stranded cDNA were synthesised, corresponding to a yield of 19% of the input RNA. Approximately 800 ng of double-stranded cDNA were obtained after synthesis of the second strand of cDNA. Following size-fractionation, the cDNAs with a size greater than 500 bp contained only 3% of the total radioactivity present in the unfractionated cDNA sample. This corresponded to approximately 25 ng of cDNA greater than 500 bp in size. Following addition of *Eco*R1 linker molecules to this size class of cDNA and ligation to λgt11 arms, a total of 1.6×10^5 bacteriophage particles were obtained, of which 60% contained recombinant DNA sequences (i.e. produced white plaques when plated in the presence of X-gal plus IPTG). This value corresponds to a cloning efficiency of 4×10^3 recombinants/μg cDNA. The *in vitro* packaging and plating efficiency of the wild-type λcI857 control DNA, was 2×10^8 p.f.u./μg DNA.

The unamplified wheat endosperm cDNA library was screened for the presence of sequences related to the wheat leaf ADP-glucose pyrophosphorylase cDNA insert, WL:AGA.1, as described in Section II.16.B. In a screen of 3×10^4 recombinant bacteriophage particles, ten positive signals were detected. Six of these clones were plaque-purified during two additional rounds of screening and these clones were designated WE:AGA.1, WE:AGA.3, WE:AGA.4, WE:AGA.5, WE:AGA.6 and WE:AGA.7. DNA was prepared from these six putative wheat endosperm ADP-glucose pyrophosphorylase cDNA clones, as described in Section II.17.B. Following restriction endonuclease digestion of the DNAs with *Eco*R1 and agarose gel electrophoresis, the sizes of the cDNA inserts of these clones were shown to range from 400 bp to 1800 bp (Table III.4).

The 1800 bp cDNA insert of clone WE:AGA.7 appeared to be sufficiently long enough to be a full-length sequence. To determine whether this was indeed the case, the denatured, glyoxalated cDNA insert was electrophoresed alongside wheat endosperm poly(A)-containing RNA, in a 1% (w/v) DMSO/glyoxal gel (Section II.18.B.). Following transfer of the nucleic acids to a nylon membrane and hybridisation of the WE:AGA.7 cDNA insert, the cDNA was shown to be the same approximate size as wheat endosperm ADP-glucose pyrophosphorylase mRNA (Figure III.23). Thus, it seemed that the cDNA insert of clone WE:AGA.7 might be full-length.

In order to further clarify the coding potential of clone WE:AGA.7, the cDNA insert was sub-cloned into the *Eco*R1 site of plasmid pBSM13$^+$ and then transcribed *in vitro*, using T_7 RNA polymerase. Following purification of WE:AGA.7 mRNA, it was translated *in vitro* in the rabbit reticulocyte cell-free translation system. The *in vitro* translation products of WE:AGA.7 mRNA were subsequently immunoprecipitated with anti-spinach leaf ADP-glucose pyrophosphorylase serum. As shown in Figure III.24, several translation products were synthesised using WE:AGA.7 mRNA as a template, suggesting that translation was initiating at more than one ATG start codon in the mRNA. Four of the polypeptides synthesised from WE:AGA.7 mRNA, were immunoprecipitated by incubation with anti-spinach leaf ADP-glucose pyrophosphorylase serum (Figure III.24, lane 6). As shown in lane 7 of Figure III.24, immunoprecipitation of these polypeptides is not detected when an excess of unlabelled, purified wheat endosperm ADP-glucose pyrophosphorylase is added to the reaction mixtures. The ability

Figure III.23: Determination of the sizes of WE:AGA.3 and WE:AGA.7 cDNA inserts compared to wheat endosperm ADP-glucose pyrophosphorylase mRNA. Poly(A)-containing RNA from wheat endosperm, or purified cDNA insert was glyoxalated and resolved on a 1% (w/v) agarose gel. Glyoxalated *Hind*III fragments of bacteriophage lambda DNA were used as size markers. The nucleic acids were transferred onto GeneScreen Plus membrane and hybridised to the nick-translated cDNA insert of clone WE:AGA.7 as described in the 'Materials and Methods' section. Lane 1 contains 0.5 μg of wheat endosperm poly(A)-containing RNA. Lane 2 contains 1 ng of purified WE:AGA.7 cDNA insert. Lane 3 contains 1 ng of purified WE:AGA.3 cDNA insert. Size marker fragment lengths are indicated on the left.

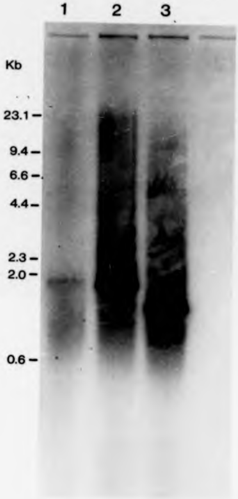
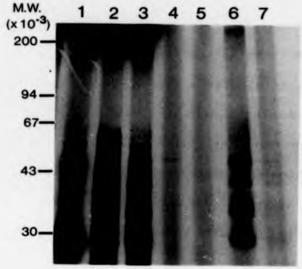

1
2
3
Kb
23.1
9.4
6.6
4.4
2.3
2.0
0.6

Figure III.24: Coupled *in vitro* transcription and translation of wheat endosperm ADP-glucose pyrophosphorylase from the cDNA insert of clone WE:AGA.7. The cDNA insert of clone WE:AGA.7 was sub-cloned into plasmid pBSM13+. Wheat endosperm ADP-glucose pyrophosphorylase mRNA was synthesised *in vitro* and then translated in the presence of L-[^{35}S] methionine as desxcribed in the 'Materials and Methods' section. Translation products were incubated with anti-spinach leaf ADP-glucose pyrophosphorylase serum and the immunoprecipitates obtained were subsequently analysed on a 10-20% (w/v) SDS/polyacrylamide gel. Lane 1 contains endogenous translation products (without added RNA). Lane 2 contains the translation products of wheat endosperm poly(A)-containing RNA extracted from free polysomes. Lane 3 contains the translation products of WE:AGA.7 mRNA. Lane 4 contains the immunoprecipitate of Lane 2 products incubated with antiserum. Lane 5 contains the immunoprecipitate of Lane 2 products incubated with antiserum plus 20 μg purified wheat endosperm ADP-glucose pyrophosphorylase. Lane 6 contains the immunoprecipitate of Lane 3 products incubated with antiserum. Lane 7 contains the immunoprecipitate of Lane 3 products incubated with antiserum plus 20 μg purified wheat endosperm ADP-glucose pyrophosphorylase. Molecular weights are indicated on the left.

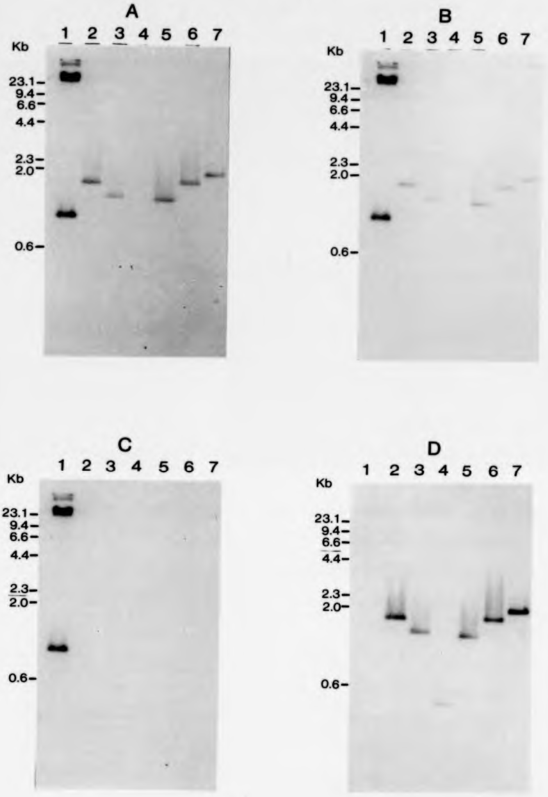

M.W.
(x 10-3)
200
94
67
43
30
1
2
3
4
5
6
7

of purified ADP-glucose pyrophosphorylase to compete with the *in vitro* translation products of WE:AGA.7 mRNA for antibody binding strongly suggests that these polypeptides contain ADP-glucose pyrophosphorylase epitopes. The synthesis of truncated ADP-glucose pyrophosphorylase polypeptides during *in vitro* translation of WE:AGA.7 mRNA may be the result of initiation at internal ATG codons which are in-frame with the ATG codon of full-length ADP-glucose pyrophosphorylase mRNA. The apparent molecular weights of the truncated ADP-glucose pyrophosphorylase polypeptides are 52,000, 42,000, 34,000 and 27,000. Only two translation products of WE:AGA.7 mRNA, with molecular weights of 39,000 and 30,000, are not immunoprecipitated by anti-spinach leaf ADP-glucose pyrophosphorylase serum, (Figure III.24, compare lane 3 and lane 6). It is possible that the syntheses of the 39 kD and 30 kD polypeptides are initiated at ATG codons which are out-of-frame with the translation start codon of full-length ADP-glucose pyrophosphorylase mRNA. The largest ADP-glucose pyrophosphorylase translation product of WE:AGA.7 mRNA has an apparent molecular weight of 52,000 and is therefore smaller than the *in vitro* translation products of wheat endosperm ADP-glucose pyrophosphorylase mRNA (Figure III.24, compare lane 3 and lane 4), indicating that the cDNA insert is probably not full-length. However,since the 52 kD *in vitro* translation product of WE:AGA.7 mRNA is larger than the mature wheat endosperm ADP-glucose pyrophosphorylase polypeptide, then clone WE:AGA.7 may contain sufficient nucleotide sequence to code for the mature wheat endosperm ADP-glucose pyrophosphorylase polypeptide.

C. Cross-hybridisation of cDNAs encoding wheat ADP-glucose pyrophosphorylases

DNA from the wheat endosperm and wheat leaf cDNA clones was digested with the restriction enzyme *Eco*R1 and electrophoresed on a 0.7% (w/v) agarose gel as described in Section II.18.A. Following transfer of the DNAs to nylon membrane, they were subsequently hybridised to the nick-translated cDNA inserts of either clone WL:AGA.1, or clone WE:AGA.7. Since the melting temperature of a DNA duplex is proportional to the ionic strength of the buffer in which it is dissolved (Maniatis *et al*, 1982), the hybridisation stringency was altered by washing membranes successively in buffers of decreasing ionic strength as described in Section II.21.C. As shown in Figure III.25A, all wheat ADP-glucose pyrophosphorylase cDNA inserts hybridise to the cDNA insert from clone WL:AGA.1 under conditions of low stringency hybridisation (i.e. when filters are washed in 2xSSC, 0.1% (w/v) SDS at 65°C). A slight increase in the hybridisation stringency, achieved by washing filters in 1xSSC, 0.1% (w/v) SDS at 65°C, resulted in the removal of approximately 50% of the ^{32}P-labelled WL:AGA.1 cDNA from the wheat endosperm cDNAs without significant reduction in the hybridisation of the wheat leaf cDNA to itself (Figure III.25B). After filters were washed in 0.5xSSC, 0.1% (w/v) SDS at 65°C, there was no apparent cross-hybridisation of the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase cDNAs (Figure III.25C). Thus, the leaf and endosperm cDNA sequences are not highly conserved. In contrast, as shown in Figure III.25D, the wheat endosperm ADP-glucose pyrophosphorylase cDNA inserts all hybridise to the nick-translated WE:AGA.7 cDNA

Figure III.25: Cross-hybridisation of wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase cDNAs. Bacteriophage DNA was restricted with *Eco*RI and fractionated on a 0.7% (w/v) agarose gel. The DNA was then transferred to Hybond N membrane and hybridised to the nick-translated cDNA insert from either clone WL:AGA.1 (Panels A, B, C) or clone WE:AGA.7 (Panel D). Following hybridisation, the membrane was washed at successively higher stringencies in the following buffers: Panel A, 2xSSC, 0.1% (w/v) SDS; Panel B, 1xSSC, 0.1% (w/v) SDS; Panel C, 0.5xSSC, 0.1% (w/v) SDS; Panel D, 0.1xSSC, 0.1% (w/v) SDS. The source of DNA in each lane is as follows: Lane 1, clone WL:AGA.1; Lane 2, clone WE:AGA.1; Lane 3, clone WE:AGA.3; Lane 4, clone WE:AGA.4; Lane 5, clone WE:AGA.5; Lane 6, clone WE:AGA.6; Lane 7, WE:AGA.7. Size marker fragment lengths are indicated on the left of each figure.


A
1 2 3 4 5 6 7
Kb
23.1
9.4
6.6
4.4
2.3
2.0
0.6
B
1 2 3 4 5 6 7
Kb
23.1
9.4
6.6
4.4
2.3
2.0
0.6
C
1 2 3 4 5 6 7
Kb
23.1
9.4
6.6
4.4
2.3
2.0
0.6
D
1 2 3 4 5 6 7
Kb
23.1
9.4
6.6
4.4
2.3
2.0
0.6

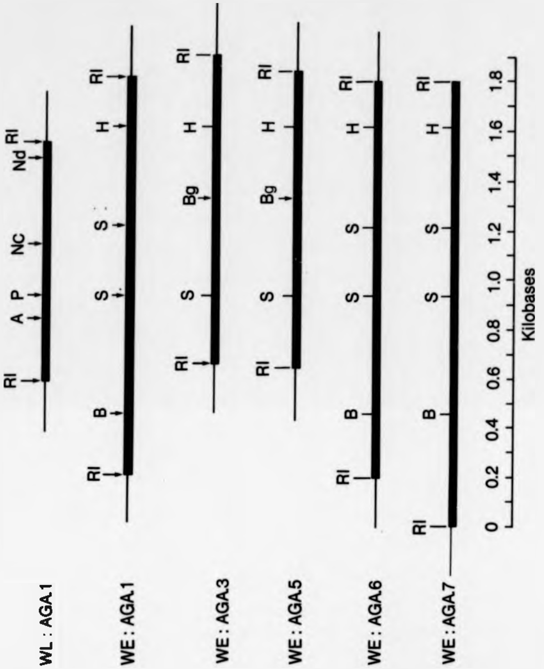
under conditions of high stringency hybridisation (i.e. filters washed in 0.1xSSC, 0.1% (w/v) SDS at 65°C), indicating that these sequences are highly conserved. Thus, on the basis of nucleic acid hybridisation data, the wheat ADP-glucose pyrophosphorylase cDNAs can be classified into two distinct sub-families containing wheat leaf sequences in one sub-family and wheat endosperm sequences in the other sub-family.

D. Restriction mapping of ADP-glucose pyrophosphorylase cDNAs

Restriction maps of the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase cDNAs were constructed to demonstrate their relatedness and also to facilitate subsequent nucleotide sequence determination. The cDNA inserts were firstly sub-cloned into the *Eco*R1 site of plasmid pBSM13+ as described in Section II.20. DNA of each sub-clone was prepared, digested with restriction enzymes and the products were analysed on 0.7% (w/v) agarose gels. As shown in Figure III.26, the restriction map of the wheat leaf ADP-glucose pyrophosphorylase cDNA is very different from the maps of wheat endosperm ADP-glucose pyrophosphorylase cDNAs, with no internal restriction enzyme sites in common. This result supports previous hybridisation analysis, which indicates that the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase cDNAs represent separate gene sub-families.

In contrast, the wheat endosperm cDNAs WE:AGA.1, WE:AGA.3, WE:AGA.5, WE:AGA.6 and WE:AGA.7 are closely related. The cDNAs all contain the unique *Hind*III site located at the 3'-terminus, and

Figure III.26: Restriction maps of ADP-glucose pyrophosphorylase cDNA inserts. The left ends of the cDNA inserts represent the 5'-end of the corresponding ADP-glucose pyrophosphorylase mRNA. The orientation of endosperm cDNAs is based on the absence of a *Bam*HI site in the shorter homologous cDNAs, which suggests that the *Bam*HI site is at the 5'-end. The orientation of the wheat leaf ADP-glucose pyrophosphorylase cDNA (WL:AGA.1) has been determined from nucleotide sequence data. The alignment of wheat endosperm and wheat leaf sequences presented here is also based on nucleotide sequence data. Enzymes employed are *Acc*I (A), *Bam*HI (B), *Bgl*II (Bg) *Eco*RI (RI), *Hind*III (H), *Nco*I (Nc), *Nde*I (Nd), *Pst*I (P), *Sst*I (S). A scale showing map distance, in kilobase pairs, is indicated at the bottom of the figure.

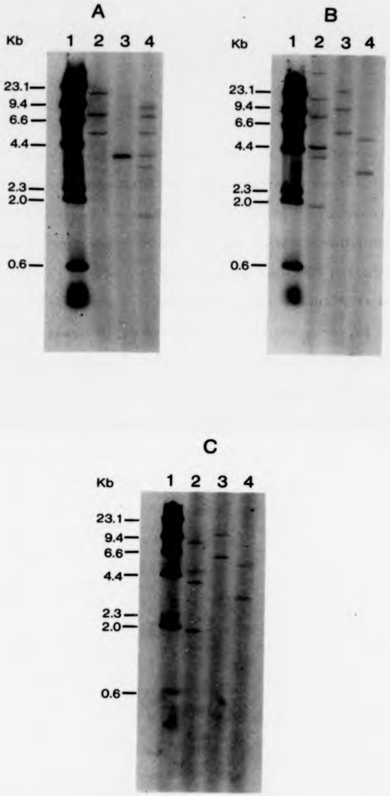

WL : AGA.1
RI
A
P
NC
Nd
RI
WE : AGA.1
RI
B
S
S
H
RI
WE : AGA.3
RI
S
Bg
H
RI
WE : AGA.5
RI
S
Bg
H
RI
WE : AGA.6
RI
B
S
S
H
RI
WE : AGA.7
RI
B
S
S
H
RI
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Kilobases

the *Sst*I site located at position -680 relative to the *Hind*III site. A unique *Bam*HI site is present in the 5'-region of the longer cDNA inserts, WE:AGA.1, WE:AGA.6 and WE:AGA.7 (Figure III.26). There is polymorphism around the *Sst*I site located at position -400 relative to the *Hind*III site of the wheat endosperm ADP-glucose pyrophosphorylase cDNAs, since this site is present only in clones WE:AGA.1, WE:AGA.6 and WE:AGA.7 (Figure III.26). In addition, the *Bgl*II site located at position -340 relative to the *Hind*III site, is only present in clones WE:AGA.3 and WE:AGA.5 (Figure III.26). These results suggest that the wheat endosperm ADP-glucose pyrophosphorylase gene sub-family may be divided into at least two distinct gene classes, with Class I represented by clones WE:AGA.3, WE:AGA.5 and Class II represented by clones WE:AGA.1, WE:AGA.6 and WE:AGA.7.

E. Organisation of wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase genes

To study the organisation of wheat ADP-glucose pyrophosphorylase genes in more detail, wheat genomic DNA was isolated and digested with restriction enzymes *Eco*RI, *Kpn*I, and *Xba*I. Following electrophoresis of the digested DNAs on a 0.7% (w/v) agarose gel, the DNA was transferred to nylon membrane and probed with the nick-translated cDNA insert from either clone WL:AGA.1 or clone WE:AGA.7. As shown in Figure III.27, there was no cross-hybridisation between wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase genes, even under low stringency hybridisation conditions. Since the wheat leaf and wheat endosperm

Figure III.27: Organisation of wheat ADP-glucose pyrophosphorylase genes. Genomic DNA from wheat was digested with restriction enzymes, fractionated on a 0.7% (w/v) agarose gel and then transferred onto nylon membrane as described in the 'Materials and Methods' section. The DNA was subsequently hybridised to the nick-translated cDNA insert from clone WL:AGA.1 (Panel A), clone WE:AGA.7 (Panel B), or clone WE:AGA.3 (Panel C). Hybridised filters were washed with 2xSSC, 0.1% (w/v) SDS. Lane 1 contains a ^{32}P-labelled *Hin*dIII digest of lambda DNA. The restriction enzymes used were as follows: Lane 2, *Eco*RI; Lane 3, *Kpn*I, Lane 4, *Xba*I. Size marker fragment lengths are indicated on the left of each figure.


A
Kb
1
2
3
4
23.1
9.4
6.6
4.4
2.3
2.0
0.6
B
Kb
1
2
3
4
23.1
9.4
6.6
4.4
2.3
2.0
0.6
C
Kb
1
2
3
4
23.1
9.4
6.6
4.4
2.3
2.0
0.6

ADP-glucose pyrophosphorylase cDNAs do cross-hybridise under low stringency conditions (Section III.5.C.), the absence of detectable cross-hybridisation of the corresponding genes is probably due to the presence of non-conserved intron sequences.

The restriction enzymes used in this analysis were chosen because the ADP-glucose pyrophosphorylase cDNAs did not contain the corresponding restriction enzyme recognition sites. Thus, based on the assumption that these restriction enzymes cleave at few sites within possible intron sequences of ADP-glucose pyrophosphorylase genes, the total number of hybridising bands provides an upper limit to the number of genes present. As shown in Figure III.27, five DNA fragments in the *Eco*RI restriction digest hybridise to the WE:AGA.7 cDNA insert under conditions of low stringency hybridisation, indicating the presence of at most five wheat endosperm ADP-glucose pyrophosphorylase genes. However, unless the wheat endosperm genes are very closely linked it is unlikely that five genes would produce only 2-3 bands on digestion of the DNA with the restriction enzymes *Kpn*I or *Xba*I. It is therefore possible that there may be as few as 2-3 wheat endosperm ADP-glucose pyrophosphorylase genes. Hybridisation of the cDNA insert from clone WE:AGA.3 to wheat genomic DNA produced the same banding pattern as did the hybridisation of WE:AGA.7 to wheat DNA, with the exception that the large 15 kb *Eco*RI fragment of wheat DNA which hybridises to WE:AGA.7 cDNA, does not hybridise to the WE:AGA.3 cDNA insert (Figure III.27). Thus, the wheat endosperm ADP-glucose pyrophosphorylase genes are organised into a small, closely related sub-family. Experiments carried out since this time indicate that both the 15 kb fragment and a 4.5 kb *Eco*RI fragment of wheat DNA hybridise to the 450 bp *Eco*RI/*Bam*HI fragment located at the 5'-terminus of clone WE:AGA.7 (result not shown). It is therefore possible that clone WE:AGA.3 does not extend far enough into the 5'-terminus of one of the ADP-glucose pyrophosphorylase genes, to hybridise to the 15 kb *Eco*RI fragment.

In restriction digests of wheat genomic DNA probed with the wheat leaf ADP-glucose pyrophosphorylase cDNA insert (WL:AGA.1), there were 3 *Eco*RI fragments, 2 *Kpn*I fragments and 6 *Xba*I fragments which hybridised under low stringency conditions (Figure III.27). Thus, the wheat leaf ADP-glucose pyrophosphorylase gene sub-family is also small, consisting of approximately 2-6 genes.

When hybridisations of cDNA inserts to wheat genomic DNA were carried out at high stringency (i.e. filters were washed in 0.1xSSC, 0.1% (w/v) SDS at 65°C), there was no selective removal of any DNA bands which had hybridised previously under low stringency conditions, to either the wheat leaf or wheat endosperm cDNA sequences (results not shown). Thus, it has not been possible to further subdivide the wheat endosperm and wheat leaf ADP-glucose pyrophosphorylase gene sub-families on the basis of hybridisation analysis. The results of genomic Southern blot hybridisations have supported the previous classification of wheat ADP-glucose pyrophosphorylase genes, obtained from Southern blot hybridisation analysis of the cDNA inserts (see Section III.5.D.).

F. Sequence analysis of wheat ADP-glucose pyrophosphorylase cDNAs and polypeptides

1. Sequencing strategy

The nucleotide sequences of the wheat leaf ADP-glucose pyrophosphorylase cDNA insert WL:AGA.1, and two wheat endosperm ADP-glucose pyrophosphorylase cDNA inserts WE:AGA.3 and WE:AGA.7, were determined as described in Section II.24. The sequencing strategies of wheat ADP-glucose pyrophosphorylase cDNAs are presented in Figure III.28. Restriction fragments of cDNA inserts were sub-cloned into the polylinker of plasmid pBSM13+ and then sequenced from both ends by taking advantage of the presence of a priming site on either side of the polylinker sequence. However, the sequence data obtained from restriction fragments alone were incomplete, because too few restriction enzyme cleavage sites were mapped (Section III.5.D.) to allow the sub-cloning of a complete 'bank' of overlapping fragments. Complete sequence data were subsequently obtained by using synthetic oligonucleotides, complementary to internal regions of the cDNA, to prime the DNA polymerase reaction. These primers were synthesised at I.C.I. Plant Protection Division, Jealott's Hill, Berkshire, U.K.

The nucleotide sequences and derived amino acid sequences of the ADP-glucose pyrophosphorylase cDNA inserts from clones WL:AGA.1, WE:AGA.3 and WE:AGA.7 are presented in Figure III.29. The derived amino acid sequences were determined from the longest open reading frames of the cDNA sequences. The identity of clones WL:AGA.1 and WE:AGA.7 as ADP-glucose pyrophosphorylase cDNAs was supported by comparisons of the amino acid sequences with the

Figure III.28: Strategy for sequencing ADP-glucose pyrophosphorylase cDNA inserts. Restriction fragments of clones WL:AGA.1, WE:AGA.3 and WE:AGA.7 were sub-cloned into the polylinker site of plasmid pBSM13+ and sequenced as described in the 'Materials and Methods' section. The sequencing reactions of plasmid subclones were primed using either the M13 -20 primer (●) or the reverse sequencing primer (■). The priming sites for these oligonucleotide primers are indicated on the figure, on either side of the polylinker region of pBSM13+. Additional nucleotide sequence data overlapping the restriction enzyme claevage sites was obtained by priming sequencing reactions with synthetic oligonucleotides, at certain positions along the cDNA insert (◆). The direction of sequencing is indicated in each case by an arrow. A scale showing map distance units in kilobase pairs is provided at the bottom of the figure.

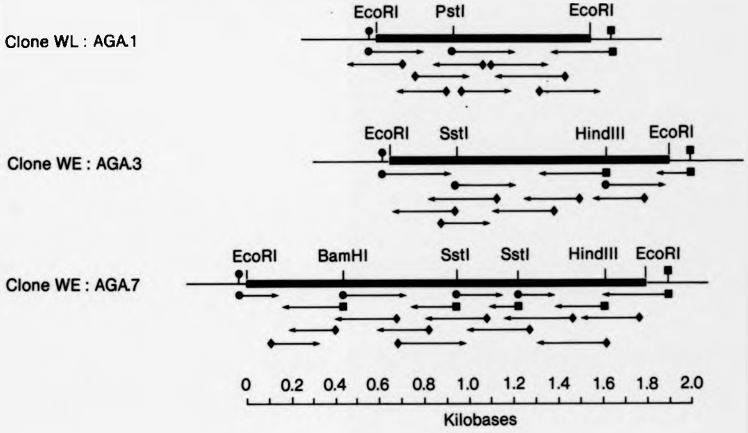
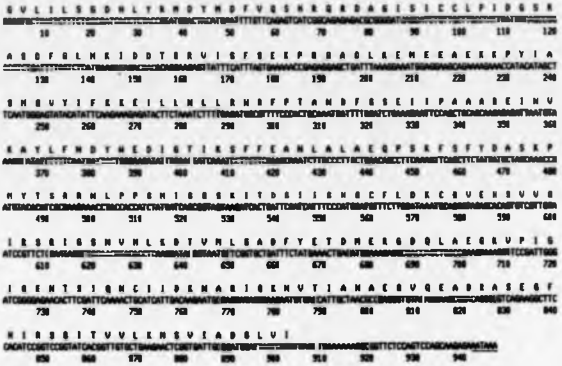

Clone WL : AGA.1
EcoRI
PstI
EcoRI
Clone WE : AGA.3
EcoRI
SstI
HindIII
EcoRI
Clone WE : AGA.7
EcoRI
BamHI
SstI
SstI
HindIII
EcoRI
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Kilobases

Figure III.29: Nucleotide sequences and derived amino acid sequences of wheat ADP-glucose pyrophosphorylase cDNA clones. The cDNA inserts of clone WL:AGA.1 (Panel A) and clones WE:AGA.3 (Panel B) and WE:AGA.7 (Panel C) were determined as described in the 'Materials and Methods' section. The amino acid sequences indicated were derived from the nucleotide sequence of the longest open reading frame in each case. The putative polyadenylation signals in each nucleotide sequence are underlined. The position of the putative processing site for the WE:AGA.7 polypeptide is indicated by an arrow.

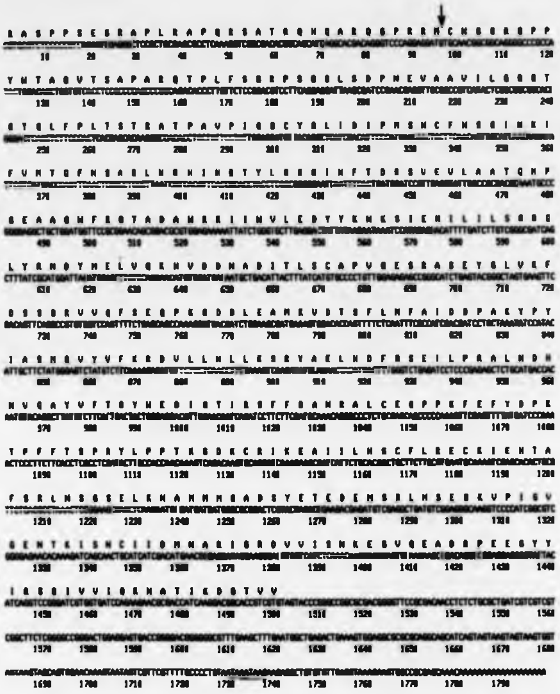
A) WL : AGA.1



B) WE : AGA.3

```
 E L V Q K N V D D N A D I T L S C A P V D E S R A S E Y Q L V E F D S S G E V V
[illegible]
     10      20      30      40      50      60      70      80      90     100     110     120

 Q F S E K P K G D D L E A M K V D T S F L N F A I D D P A K Y P Y I A S M G V Y
TCCAA[illegible]TAAATATCCATACATTGCTTCTATGGGAGTCT
    130     140     150     160     170     180     190     200     210     220     230     240

 V F K R D V L L N L L E S R Y A E L H D F G S E I L P R A L H D H N V Q A Y V F
ATGTCTT[illegible]ACGCAGAACTACATGACTTTGGGTCTGAAATCCTCCCGAGAGCTCTGCATGACCACAATGTACAGGCTTATGTCT
    250     260     270     280     290     300     310     320     330     340     350     360

 T D Y N E D I Q T I Q E F F D A N H S L C E Q P P K F E F Y D P K T P F F T S P
TCACT[illegible]GATGCGAACATGTCCCTCTGCGAGCAGCCCCCAAAGTTCGAGTTTTATGATCCCAAAA[illegible]
    370     380     390     400     410     420     430     440     450     460     470     480

 R Y L P P T K N Q K C R I K E A I I L N Q C F L R E C K I E N R I I Q V P S R L
CTC[illegible]
    490     500     510     520     530     540     550     560     570     580     590     600

 N R G S E L K N A N N N G A D S Y E T E Q E I S R L M E I Q K V P I Q V R E N T
TAAACTCC[illegible]
    610     620     630     640     650     660     670     680     690     700     710     720

 K I S N C I I D N N A R I G R D V V I S N K E G V Q E A D R P E E G Y Y I R S G
[illegible]ACTACATCAGGTCCG
    730     740     750     760     770     780     790     800     810     820     830     840

 I V V I Q K N A T I K D Q T V V
GGATCGTGGTGATCCAGAAGAACGCGACCATCAAGGAC[illegible]GTAGTACCC[illegible]TCGTCGTCTCGAG
    850     860     870     880     890     900     910     920     930     940     950     960

GCCCGGGAGGGACTGAAGAAGTGACCGGGGACGGGAGGCGTTTGAAGCTTGAATGACTGAGAAGGCGCGCGCGGGCAGCATTAGTAGTAAGTAGTAGTAAGGAGCAGTGGAACAAAGTAA
    970     980     990    1000    1010    1020    1030    1040    1050    1060    1070    1080

TAGTCGTTCGTTTTTCCCCTGTAATAAATAAGAGGCTGTGTGTTGAGGTAAAGAAGTGGCAGCGAGCAAACAAACTCCCGGGGGATGTTCGTGTAAATAAAACTCTATCTAGACCTGTGA
   1090    1100    1110    1120    1130    1140    1150    1160    1170    1180    1190    1200

AATTTTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
   1210    1220    1230    1240    1250    1260    1270
```

C) WE : AGA.7



amino acid sequence derived from a rice endosperm ADP-glucos pyrophosphorylase cDNA clone (Preiss *et al*, 1987). In the fi amino acids residues encoded by clone WL:AGA.1 there is approximately 70% identity with residues 37-63 of the rice endosperm amino acid sequence. Between amino acid residues 1 encoded by clone WE:AGA.7 there is 65% identity with residu of the rice endosperm sequence. In addition, there is approximately 50% identity between amino acids 62-119 encode clone WE:AGA.7 and the fructose-1,6-bisphosphate binding sit *E.coli* ADP-glucose pyrophosphorylase (Parsons and Preiss, 1 Regions of identity between wheat and other ADP-glucose pyrophosphorylase polypeptides are discussed more completel Section III.5.F.7 and Section III.5.G (see also Figure III.

2. Primary structure of cDNA clone WL:AGA.1

The cDNA insert from clone WL:AGA.1 (Figure III.29A) is in length and encodes 301 amino acids at the C-terminus of leaf ADP-glucose pyrophosphorylase. These 301 amino acids p a polypeptide with a molecular weight of 33,400. Thus, approximately 70% of the mature wheat leaf ADP-glucose pyrophosphorylase polypeptide sequence is encoded by clone WL:AGA.1. The cDNA extends 45 nucleotides into the 3'-untra region of the corresponding mRNA and terminates at the puta polyadenylation signal AATAAA (Joshi, 1987b), located at po 942-947. Consequently, there is no poly(A) tail in this whe ADP-glucose pyrophosphorylase cDNA sequence.

Codon usage in the wheat leaf ADP-glucose pyrophosphoryl cDNA was calculated using the programme of Staden (1982). A

Figure III.30: Codon usage in WL:AGA.1 and WE:AGA.7 cDNAs. Codon usage for the cDNAs encoding (A) wheat leaf ADP-glucose pyrophosphorylase (i.e. clone WL:AGA.1) and (B) wheat endosperm ADP-glucose pyrophosphorylase (i.e. clone WE:AGA.7) were calculated using the programme of staden (1982). The absolute number of times a particular codon is utilised in a given sequence is indicated.

A) Clone WL : AGA.1

Residue	Codon	No.	Residue	Codon	No.	Residue	Codon	No.	Residue	Codon	No.
F	TTT	5	S	TCT	5	Y	TAT	3	C	TGT	2
F	TTC	9	S	TCC	4	Y	TAC	6	C	TGC	3
L	TTA	2	S	TCA	4	*	TAA	0	*	TGA	1
L	TTG	3	S	TCG	4	*	TAG	0	W	TGG	2
L	CTT	6	P	CCT	2	H	CAT	2	R	CGT	3
L	CTC	4	P	CCC	1	H	CAC	4	R	CGC	0
L	CTA	2	P	CCA	4	Q	CAA	1	R	CGA	2
L	CTG	3	P	CCG	3	Q	CAG	5	R	CGG	3
I	ATT	12	T	ACT	5	N	AAT	7	S	AGT	4
I	ATC	11	T	ACC	1	N	AAC	6	S	AGC	5
I	ATA	8	T	ACA	2	K	AAA	6	R	AGA	7
M	ATG	9	T	ACG	2	K	AAG	12	R	AGG	3
V	GTT	5	A	GCT	7	D	GAT	14	G	GGT	5
V	GTC	2	A	GCC	3	D	GAC	8	G	GGC	5
V	GTA	6	A	GCA	6	E	GAA	13	G	GGA	9
V	GTG	4	A	GCG	6	E	GAG	6	G	GGG	5

Total codons = 302
WL : AGA.1 polypeptide molecular weight = 33430
WL : AGA.1 polypeptide hydrophobicity = -11.26

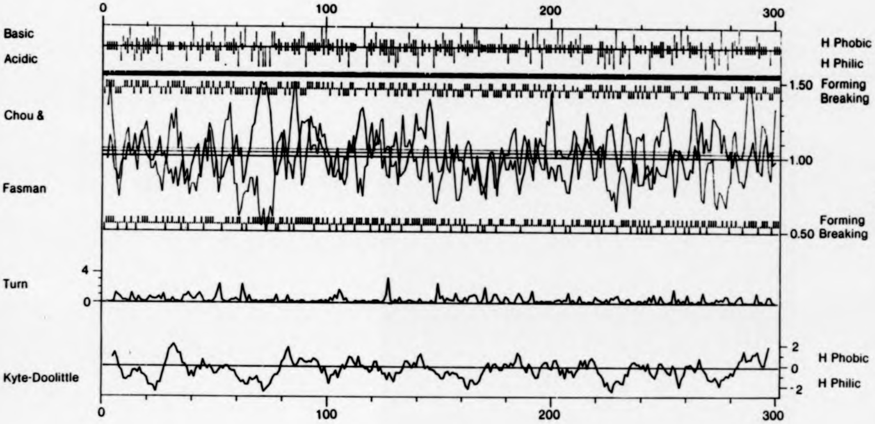
B) Clone WE : AGA.7

Residue	Codon	No.	Residue	Codon	No.	Residue	Codon	No.	Residue	Codon	No.
F	TTT	4	S	TCT	5	Y	TAT	8	C	TGT	2
F	TTC	18	S	TCC	8	Y	TAC	11	C	TGC	7
L	TTA	2	S	TCA	8	*	TAA	0	*	TGA	0
L	TTG	6	S	TCG	8	*	TAG	1	W	TGG	5
L	CTT	5	P	CCT	7	H	CAT	4	R	CGT	5
L	CTC	10	P	CCC	9	H	CAC	6	R	CGC	4
L	CTA	4	P	CCA	8	Q	CAA	3	R	CGA	3
L	CTG	6	P	CCG	6	Q	CAG	13	R	CGG	3
I	ATT	9	T	ACT	6	N	AAT	7	S	AGT	3
I	ATC	20	T	ACC	9	N	AAC	14	S	AGC	6
I	ATA	4	T	ACA	9	K	AAA	9	R	AGA	5
M	ATG	14	T	ACG	2	K	AAG	14	R	AGG	14
V	GTT	5	A	GCT	10	D	GAT	10	G	GGT	3
V	GTC	11	A	GCC	10	D	GAC	20	G	GGC	15
V	GTA	2	A	GCA	4	E	GAA	6	G	GGA	15
V	GTG	11	A	GCG	14	E	GAG	23	G	GGG	8

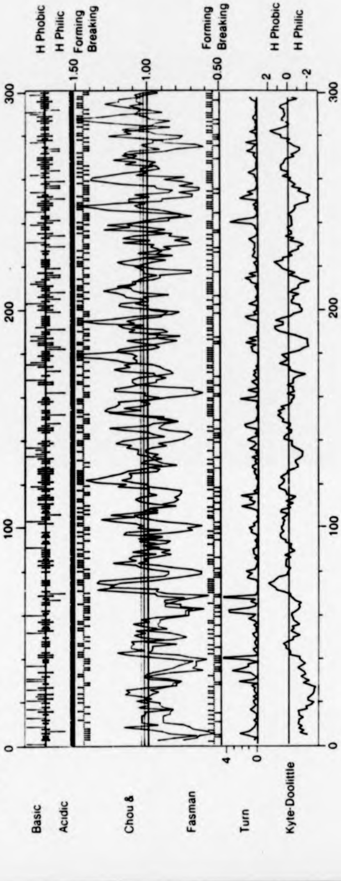
Total codons = 501
WE : AGA.7 polypeptide molecular weight = 55560
WE : AGA.7 polypeptide hydrophobicity = -35.80

Figure III.31: Secondary structure predictions and hydropathy profiles of the WL:AGA.1 and WE:AGA.7 derived polypeptides. The amino acid sequences of clone WL:AGA.1 (Panel A) and clone WE:AGA.7 (Panel B) were analysed using the programme of Devereux *et al* (1987). In the top box is a representation of the amino acid sequence: Green lines are hydrophilic charged amino acids; lines pointing down are aspartate or glutamate residues; lines pointing up are lysine, arginine, or histidine residues. Short red lines are uncharged amide residues. Long red lines indicate uncharged, hydroxylated residues. Short blue lines are aliphatic amino acids. Long blue lines indicate aromatic residues. Proline residues are shown in black. Alanine, glycine and cysteine residues are unmarked. The second part of the plot shows the Chou and Fasman (1978) predictions for the formation of α-helix (red line) and β-sheet (blue line) secondary structures. Above the two curves is a display of the residues that are β-sheet forming and breaking. Below the two curves is a display of the residues that are α-helix forming and breaking. The horizontal red line shows the threshold Chou and Fasman (1978) propensity measure for formation of an α-helix structure. The horizontal blue line shows the Chou and Fasman (1978) threshold propensity for formation of a β-sheet structure. The third part of the plot shows the probability (x 1000) that a particular sequence will be found in a β-turn. The Chou and Fasman (1978) parameters have been calculated over a window of four residues. The fourth part of the plot shows the Kyte and Doolittle (1982) hydropathy measure, calculated as the average of a residue-specific hydrophobicity index over a window of nine residues.

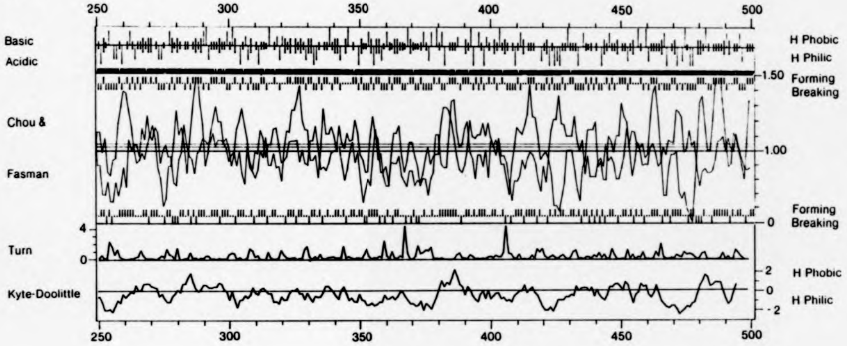
A) Clone WL : AGA.1



B) Clone WE : AGA.7



B) Clone WE : AGA.7



in Figure III.30A, there are no significant codon preferences in WL:AGA.1, possibly because the cDNA clone is too small for any preferences to become evident. The TGA stop codon is present in the WL:AGA.1 cDNA.

The hydropathy index (Kyte and Doolittle, 1982) and secondary structure predictions (Chou and Fasman, 1978) were calculated for the WL:AGA.1 protein using the programmes of Devereux *et al* (1987), and the results are presented in Figure III.31A. The Kyte and Doolittle (1982) hydropathy profile of the WL:AGA.1 protein contains several hydrophilic regions, in addition, there are three strongly hydrophobic regions (around positions 30-40, 80-85 and at the C-terminus of the protein) which are probably located in the interior of the polypeptide (Figure III.31A). According to Kyte and Doolittle (1982), a 19-residue amino acid segment with a calculated hydrophobicity of less than +1.6, is likely to be an internal region of the protein rather than associated with bilipid membranes. The absence of long stretches of hydrophobic amino acids from the derived amino acid sequence of the WL:AGA.1 polypeptide is thus consistent with the location of ADP-glucose pyrophosphorylase in the soluble fraction of chloroplasts (Kaiser and Bassham, 1979b). The secondary structure predictions of Chou and Fasman (1978) have been used in comparisons of the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase proteins (see Section III.5.F.6).

3. Primary structure of cDNA clone WE:AGA.3

The cDNA insert from clone WE:AGA.3 (Figure III.29B) is 1272 bp in length, which includes a poly(A) tail of 65 nucleotides at the

3'-terminus. The 3'-untranslated region of WE:AGA.3 cDNA, from the TAG stop codon to the start of the poly(A) tail, is 317 bp in length. There are two overlapping polyadenylation signals present in the 317 bp 3'untranslated region of WE:AGA.3 cDNA; the sequence AATAAA is located 104 bp upstream of the polyadenylation site, while the sequence AATAAG is 100 bp upstream of the polyadenylation site (Figure III.29B). Whilst the sequence motif AATAAA is the most common found in plant mRNAs, the sequence AATAAG has also been identified in several genes including maize zein, soybean lectin, castor bean lectin, maize glutathione-S-transferase I, potato proteinase inhibitor II and carrot extensin (cited in Joshi, 1987b). Clone WE:AGA.3 also contains a third putative polyadenylation signal, AATAAA at position 1176-1181, 31 bp upstream from the polyadenylation site. Since the location of polyadenylation signals in plant mRNAs is usually 22-36 nucleotides upstream of the polyadenylation site (Joshi, 1987), then this third polyadenylation signal in clone WE:AGA.3 is the signal most likely to be involved with selection of the poly(A) addition site during processing of the corresponding ADP-glucose pyrophosphorylase mRNA. The open reading frame of clone WE:AGA.3 is 890 bp in length, encoding 296 amino acids of wheat endosperm ADP-glucose pyrophosphorylase with a total molecular weight of 33,200. This truncated polypeptide is 65% of the size of the mature wheat endosperm ADP-glucose pyrophosphorylase subunit.

4. Primary structure of cDNA clone WE:AGA.7

The largest wheat endosperm ADP–glucose pyrophosphorylase cDNA, WE:AGA.7 is 1798 bp in length, comprising an open reading frame of

1500 bp (Figure III.29C). This open reading frame codes for a truncated ADP-glucose pyrophosphorylase polypeptide of 55,500 molecular weight. This molecular weight exceeds the estimated molecular weight of the mature wheat endosperm ADP-glucose pyrophosphorylase polypeptide by approximately 4,500. It is possible therefore, that the WE:AGA.7 protein contains the complete sequence of the mature protein plus some of the amyloplast transit peptide sequence at the N-terminus.

In addition, clone WE:AGA.7 contains a 278 bp 3'-untranslated region, from the TAG stop codon to the start of the poly(A) tail (Figure III.29C). The untranslated region contains two overlapping putative polyadenylation signals, AATAAA at position 1728-1733 and AATAAG at position 1732-1737. These polyadenylation signals are located 45 bp and 41 bp respectively from the polyadenylation site and are the same sequences present in clone WE:AGA.3. It is not possible at present to determine which of these signals may be functional.

Codon usage in the wheat endosperm ADP-glucose pyrophosphorylase cDNA WE:AGA.7 has been tabulated using the programme of Staden (1982). This is shown in Figure III.30B. Deviations from random usage were found in the open reading frame of WE:AGA.7 for the codons of phenylalanine (preference = TTC), glutamine (preference = CAG) and glutamate (preference = GAG). The TAG stop codon is present in the WE:AGA.7 (and WE:AGA.3) cDNA sequence (Figure III.30B).

The Kyte and Doolittle (1982) hydropathy index and the Chou and Fasman (1978) secondary structure predictions were calculated for the WE:AGA.7 encoded polypeptide using the programme of Devereux *et al* (1987), and the results are presented in Figure III.31B. The

Kyte and Doolittle (1982) hydropathy profile of the WE:AGA.7 polypeptide is generally hydrophilic, with short hydrophobic stretches between residues 70-80, residues 190-200, residues 220-225, residues 280-290, residues 380-390 and residues 480-490. The Kyte and Doolittle (1982) hydropathy profile for the WE:AGA.7 polypeptide is entirely consistent with the location of ADP-glucose pyrophosphorylase in the soluble fraction of wheat endosperm extracts.

5. Comparison of WE:AGA.3 and WE:AGA.7 sequences

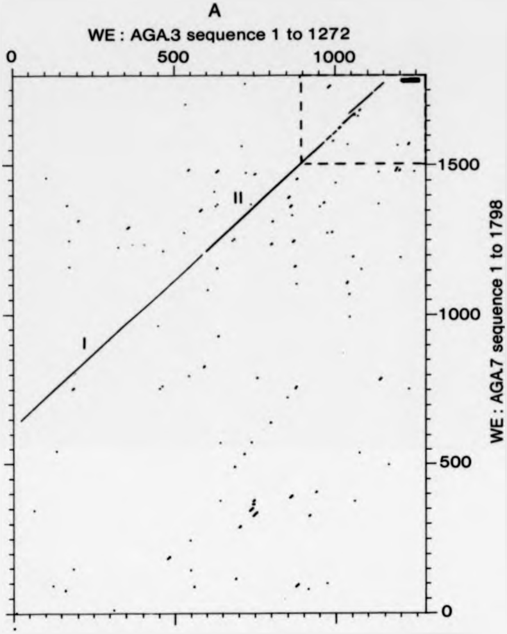
The wheat endosperm ADP-glucose pyrophosphorylase cDNA inserts of clones WE:AGA.3 and WE:AGA.7 are 96.3% identical in the shared regions of their open reading frames. In the 3'-untranslated region of these clones, from the TAG stop codons to the polyadenylation site, the extent of relatedness is reduced to 72.3%. A detailed comparison of the nucleotide sequences of WE:AGA.3 and WE:AGA.7 cDNAs reveals a total of 30 base substitutions, of which 72% are transitions. Within the open reading frame there are only 18 base substitutions, 11 transitions and 7 transversions. It is noteworthy that all of the transitions are located in third base positions of codons and none of them leads to an amino acid substitution. One of the transversions, located at position 1296 in WE:AGA.7 (Figure III.29C) and at position 686 in WE:AGA.3 (Figure III.29C), is silent. The remaining six transversions produce three conservative (threonine-serine, glutamine-lysine, alanine-serine) and two semi-conservative (arginine-methionine, isoleucine- methionine) amino acid substitutions. In addition, there are only 9 nucleotide insertions/deletions in the open reading frame, occurring at positions 579-585 and positions 592-593 of the WE:AGA.3 sequence (Figure III.29B). These insertions/deletions produce an additional 5 amino acid changes between WE:AGA.3 and WE:AGA.7 proteins, three insertions and 2 substitutions. Thus, the derived amino acid sequences of the wheat endosperm ADP-glucose pyrophosphorylase cDNAs differ in only 10 of 296 amino acid residues, yielding an amino acid identity of 96.7%.

In the 3'-untranslated region there are a total of 85 insertions/deletions between WE:AGA.3 and WE:AGA.7 sequences, clustered into 8 variable regions. Regions containing insertions or deletions between the two wheat endosperm cDNA sequences have been highlighted using the DIAGON programme of Devereux *et al* (1987), as shown in Figure III.32A.

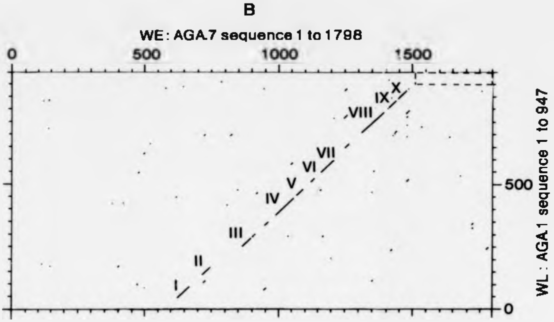
6. Comparison of WE:AGA.7 and WL:AGA.1 sequences

The wheat leaf ADP-glucose pyrophosphorylase cDNA sequence (WL:AGA.1) and the wheat endosperm ADP-glucose pyrophosphorylase cDNA sequence (WE:AGA.7) were compared using the DIAGON programme of Devereux *et al* (1987). As shown in Figure III.32B, there are 10 well-conserved domains between the two nucleotide sequences. Between WL:AGA.1 and WE:AGA.7, there are 322 nucleotide substitutions, 290 of which are within the open reading frame, plus 148 insertions/deletions. Thus, within the open reading frames for which the WL:AGA.1 and WE:AGA.7 cDNA sequences overlap, there is only 55.7% identity at the DNA level. There is no relatedness between WE:AGA.7 and WL:AGA.1 cDNA sequences in their 3'-untranslated regions.

Figure III.32: Dot matrix comparisons of wheat ADP-glucose pyrophosphorylase cDNAs. DNA sequences were compared over a moving window of 21 nucleotides and identity scored if there was identity in 14 of the 21 nucleotides in any window. In Panel A, the sequences of clones WE:AGA.3 (abscissa) and WE:AGA.7 (ordinate) were compared. In Panel B, the sequences of clones WE:AGA.7 (abscissa) and WL:AGA.1 (ordinate) were compared. The 3'-untranslated regions of cDNA sequences are indicated by broken lines.



Domain	Position of domain in nucleotide sequence	
	WE : AGA3	WE : AGA7
I	1 - 584	620 - 1203
II	594 - 911	1204 - 1521



Domain	Position of domain in nucleotide sequence	
	WE : AGA.7	WL : AGA.1
I	578 - 639	5 - 66
II	672 - 723	97 - 150
III	829 - 876	226 - 272
IV	961 - 1031	355 - 425
V	1048 - 1074	442 - 468
VI	1137 - 1155	531 - 549
VII	1159 - 1183	553 - 577
VIII	1293 - 1373	697 - 776
IX	1399 - 1425	802 - 828
X	1439 - 1473	842 - 876

To determine the relatedness between WE:AGA.7 and WL:AGA.1 encoded polypeptides, the derived amino acid sequences were aligned (Figure III.33). Of the 290 nucleotide substitutions within the open reading frame, 97 are silent. There are a total of 110 amino acid alterations between the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase polypeptide sequences, produced by the remaining 193 nucleotide substitutions and 91 of the 148 insertions/deletions. The data indicate that, in most cases nucleotide substitutions between WL:AGA.1 and WE:AGA.7 cDNA sequences involve all three nucleotide positions of codons. Thus, there is only 55.3% identity between the derived amino acid sequences of these cDNAs.

Of the 110 amino acid substitutions between wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase polypeptide sequences, 62 are conservative changes involving either no charge difference or substitution of charged residues for neutral amides (i.e. glutamine, asparagine), substitution of amphipathic residues for other amphipathic residues, or substitution of hydrophobic residues for other hydrophobic residues. There are 39 semi-conservative amino acid substitutions, involving reversal of charge, or substitution of amphipathic amino acids for charged, neutral, or hydrophobic residues. Non-conservative amino acid sustitutions, of which there are 9, involve substitution of hydrophobic residues for either charged or neutral amino acids. Two of the non-conservative amino acid substitutions (His_{512}-Tyr_{512}; Leu_{521}-Gln_{521}; Figure III.33) and two semi-conservative substitutions (Ser_{508}-Glu_{508}; Ala_{527}-Lys_{527}; Figure III.33) between wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase polypeptides are located in close proximity to,

Figure III.33: Aligned amino acid sequences of wheat leaf, wheat endosperm, rice endosperm and *E.coli* ADP-glucose pyrophosphorylase polypeptides. The derived amino acid sequences of wheat clones WL:AGA.1, WE:AGA.3, WE:AGA.7, the amino acid sequences derived from a cDNA encoding rice endosperm ADP-glucose pyrophosphorylase (Preiss *et al*, 1987) and the derived amino acid sequence of the *E.coli* glgC gene (Baecker *et al*,1983) were aligned manually. Peptide segments labelled I, II, III, IV, or V are explained in the text. Amino acids which are conserved between wheat leaf, wheat endosperm and rice endosperm sequences are indicated with an asterisk. Amino acids which are conserved in all the sequences are boxed.

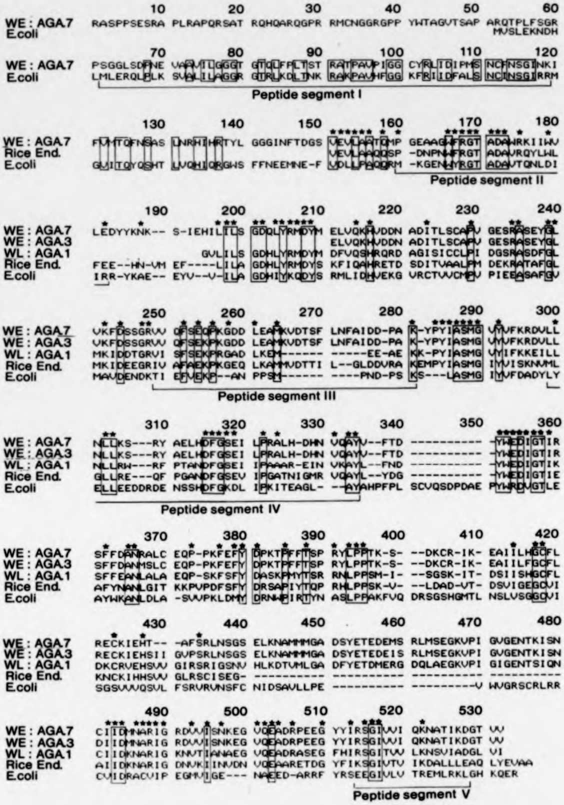

10 20 30 40 50 60
WE : AGA7 RASPPSESRA PLRAPQRSAT RQHQARQGPR RMCNGGRGPP YWTAGVTSAP ARQTPLFSGR
E.coli MVSLEKNDH
70 80 90 100 110 120
WE : AGA7 PSGGLSDFNE VAAVILGGGT GTQLFPLTST RATPAVPIGG CYRLIDIPMS NCFNSGINKI
E.coli LMLERQLPLK SVALILAGGR GTRLKDLTNK RAKPAVHFGG KFRIIDFALS NCINSGIRRM
Peptide segment I
130 140 150 160 170 180
WE : AGA7 FVMTQFNSAS LNRHIHRTYL GGGINFTDGS VEVLAATQMP GEAAGWFRGT ADAWRKIIWV
Rice End. VEVLAAQQSP -DNPNWFRGT ADAVRQYLWL
E.coli GVITQYQSHT LVQHIQRGWS FFNEEMNE-F VDLLPAQQRM -KGENWYRGT ADAVTQNLDI
Peptide segment II
190 200 210 220 230 240
WE : AGA7 LEDYYKNK-- S-IEHILILS GDQLYRMDYM ELVQKHVDDN ADITLSCAPV GESRASEYGL
WE : AGA3 ELVQKHVDDN ADITLSCAPV GESRASEYGL
WL : AGA1 GVLILS GDHLYRMDYM DFVQSHRQRD AGISICCLPI DGSRASDFGL
Rice End. FEE--HN-VM EF----LILA GDHLYRMDYS KFIQAHRETD SDITVAALPM DEKRATAFGL
E.coli IRR-YKAE-- EYV--V-ILA GDHIYKQDYS RMLIDHVEKG VRCTVVCMPV PIEEASAFGV
250 260 270 280 290 300
WE : AGA7 VKFDSSGRVV QFSEQPKGDD LEAMKVDTSF LNFAIDD-PA K-YPYIASMG VYVFKRDVLL
WE : AGA3 VKFDSSGRVV QFSEKPKGDD LEAMKVDTSF LNFAIDD-PA K-YPYIASMG VYVFKRDVLL
WL : AGA1 MKIDDTGRVI SFSEKPRGAD LKEM------ -----EE-AE KK-PYIASMG VYIFKKEILL
Rice End. MKIDEEGRIV AFAEKPKGEQ LKAMMVDTTI L--GLDDVRA KEMPYIASMG IYVISKNVML
E.coli MAVDENDKTI EFVEKP--AN PPSM------ ----PND-PS KS---LASMG IYVFDADYLY
Peptide segment III
310 320 330 340 350 360
WE : AGA7 NLLKS---RY AELHDFGSEI LPRALH-DHN VQAYV--FTD ---------- -YWEDIGTIR
WE : AGA3 NLLKS---RY AELHDFGSEI LPRALH-DHN VQAYV--FTD ---------- -YWEDIGTIR
WL : AGA1 NLLRW---RF PTANDFGSEI IPAAAR-EIN VKAYL--FND ---------- -YWEDIGTIK
Rice End. GLLRE---QF PGANDFGSEV IPGATNIGMR VQAYL--YDG ---------- -YWEDIGTIE
E.coli ELLEEDDRDE NSSHDFGKDL IPKITEAGL- --AYAHPFPL SCVQSDPDAE PYWRDVGTLE
Peptide segment IV
370 380 390 400 410 420
WE : AGA7 SFFDANRALC EQP-PKFEFY DPKTPFFTSP RYLPPTK-S- --DKCR-IK- EAIILHGCFL
WE : AGA3 SFFDANMSLC EQP-PKFEFY DPKTPFFTSP RYLPPTK-S- --DKCR-IK- EAIILFGCFL
WL : AGA1 SFFEANLALA EQP-SKFSFY DASKPMYTSR RNLPPSM-I- --SGSK-IT- DSIISHGCFL
Rice End. AFYNANLGIT KKPVPDFSFY DRSAPIYTQP RHLPPSK-V- --LDAD-VT- DSVIGEGCVI
E.coli AYWKANLDLA -SVVPKLDMY DRNWPIRTYN ASLPPAKFVQ DRSGSHGMTL NSLVSGGCVI
430 440 450 460 470 480
WE : AGA7 RECKIEHT-- -AFSRLNSGS ELKNAMMMGA DSYETEDEMS RLMSEGKVPI GVGENTKISN
WE : AGA3 RECKIEHSII GVPSRLNSGS ELKNAMMMGA DSYETEDEIS RLMSEGKVPI GVGENTKISN
WL : AGA1 DKCRVEHSVV GIRSRIGSNV HLKDTVMLGA DFYETDMERG DQLAEGKVPI GIGENTSIQN
Rice End. KNCKIHHSVV GLRSCISEG- ---------- ---------- ---------- ----------
E.coli SGSVVVQSVL FSRVRVNSFC NIDSAVLLPE ---------- ---------V WVGRSCRLRR
490 500 510 520 530
WE : AGA7 CIIDMNARIG RDVVISNKEG VQEADRPEEG YYIRSGIVVI QKNATIKDGT VV
WE : AGA3 DIIDMNARIG RDVVISNKEG VQEADRPEEG YYIRSGIVVI QKNATIKDGT VV
WL : AGA1 CIIDKNARIG KNVTIANAEG VQEADRASEG FHIRSGITVV LKNSVIADGL VI
Rice End. AIIDKNARIG DNVKIINVDN VQEAARETDG YFIKSGIVTV IKDALLLEAQ LYEVAA
E.coli CVIDRACVIP EGMVIGE--- NAEED-ARRF YRSEEGIVLV TREMLRKLGH KQER
Peptide segment V

Figure III.34: Comparison of the secondary structure predictions for the 3-phosphoglycerate binding sites of wheat leaf and wheat endosperm ADP-glucose pyrophosphorylases. The amino acid sequences of wheat ADP-glucose pyrophosphorylases related to the putative 3-phosphoglycerate binding site of spinach leaf ADP-glucose pyrophosphorylase (J. Preiss, personal communications) were aligned. The Chou and Fasman (1978) secondary structure predictions for these sequences were subsequently calculated using the programme of Devereux *et al* (1987). The amino acid numbering refers to the position of these sequences relative to the first amino acid encoded by clone WE:AGA.7, as shown in Figure III.33.

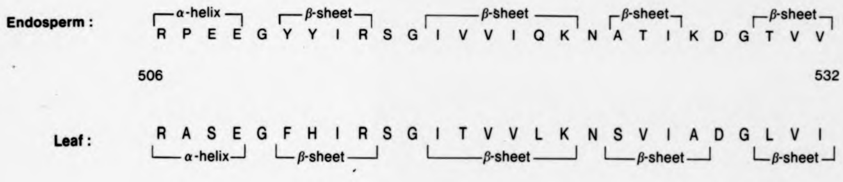
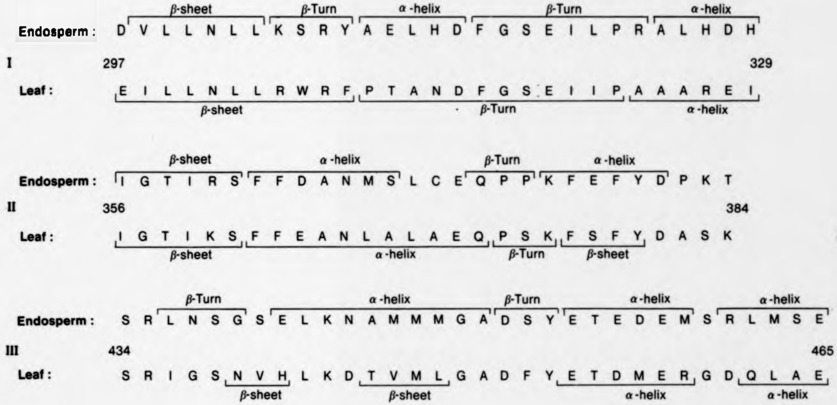

Endosperm :
α-helix
β-sheet
β-sheet
β-sheet
β-sheet
R P E E G Y Y I R S G I V V I Q K N A T I K D G T V V
506
532
Leaf :
R A S E G F H I R S G I T V V L K N S V I A D G L V I
α-helix
β-sheet
β-sheet
β-sheet
β-sheet

Figure III.35: Regions of non-conserved secondary structure between wheat leaf and wheat endosperm ADP-glucose pyrophosphorylases. The Chou and Fasman (1978) secondary structure predictions for the amino acid sequences encoded by clones WL:AGA.1 and WE:AGA.7 were calculated using the programme of Devereux *et al* (1987). These predictions were then aligned to highlight the regions for which non-conserved amino acid sequences produce a considerable change in the predicted secondary structure for the two polypeptides.


β-sheet
β-Turn
α-helix
β-Turn
α-helix
Endosperm :
D V L L N L L K S R Y A E L H D F G S E I L P R A L H D H
I
297
329
Leaf :
E I L L N L L R W R F P T A N D F G S E I I P A A A R E I
β-sheet
β-Turn
α-helix
β-sheet
α-helix
β-Turn
α-helix
Endosperm :
I G T I R S F F D A N M S L C E Q P P K F E F Y D P K T
II
356
384
Leaf :
I G T I K S F F E A N L A L A E Q P S K F S F Y D A S K
β-sheet
α-helix
β-Turn
β-sheet
β-Turn
α-helix
β-Turn
α-helix
α-helix
Endosperm :
S R L N S G S E L K N A M M M G A D S Y E T E D E M S R L M S E
III
434
465
Leaf :
S R I G S N V H L K D T V M L G A D F Y E T D M E R G D Q L A E
β-sheet
β-sheet
α-helix
α-helix

or within the region related to the putative 3-phosphoglycerate binding site on spinach leaf ADP-glucose pyrophosphorylase (J.Preiss, personal communications). However, within the C-terminal 27 residues of wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase enzymes the Chou and Fasman (1978) predictions are nearly identical (Figure III.34).

The Chou and Fasman (1978) secondary structure predictions for wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase proteins differ in three regions. The locations of these sequences and their predicted secondary structures are presented in Figure III.35. The significance of this finding is not clear however, since the function of these peptide sequences have not been elucidated.

7. Comparison of wheat and other ADP-glucose pyrophosphorylase polypeptides

The amino acid sequences derived from the wheat ADP-glucose pyrophosphorylase cDNAs were aligned with the amino acid sequences derived from a rice endosperm ADP-glucose pyrophosphorylase cDNA (Preiss *et al*, 1987) and the *E.coli* glgC gene (Baecker *et al*, 1983). This 5-way protein sequence alignment is presented in Figure III.33. The identity between amino acid sequences were subsequently calculated on the basis of this alignment as the proportion of identical residues in the overlapping regions and do not therefore take into account the size of gaps between the overlaps. There is 40% identity between the wheat endosperm ADP-glucose pyrophosphorylase sequences (WE:AGA.3 and WE:AGA.7) and rice endosperm ADP-glucose pyrophosphorylase. The wheat leaf sequence is 44% identical to rice endosperm ADP-glucose pyrophosphorylase. There is a 40 amino acid insertion in the wheat ADP-glucose pyrophosphorylase sequences, compared to the rice sequence (residues 441-480, Figure III.33). The identities between wheat and rice sequences are only slightly less than the 55% identity between wheat leaf and wheat endosperm sequences. In addition, there is 29.5% identity between the rice endosperm and *E.coli* amino acid sequences, compared to only 24% identity between the wheat leaf or wheat endosperm ADP-glucose pyrophosphorylases and *E.coli* ADP-glucose pyrophosphorylase. However, as noted previously (Section III.5.F.1) there are regions within these polypeptides which are highly conserved. For example, peptide segment I (figure III.33) which is the fructose-1,6-bisphosphate binding peptide of *E.coli* ADP-glucose pyrophosphorylase has 50% identity to the aligned region of the wheat endosperm enzyme. Additional highly conserved regions of rice, wheat and *E.coli* ADP-glucose pyrophosphorylase polypeptides are indicated in Figure III.33.

G. DISCUSSION

1. Isolation and characterisation of wheat ADP-glucose pyrophosphorylase cDNAs

The isolation and characterisation of a family of seven wheat ADP-glucose pyrophosphorylase cDNA clones is reoprted in this investigation. Clones designated WL:AGA.1 and WE:AGA.7 were shown by three independent criteria to encode, respectively, wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase:

(1). Clone WL:AGA.1 expresses ADP-glucose pyrophosphorylase antigen under the direction of the lacZ gene promoter, detected by the binding of anti-spinach leaf ADP-glucose pyrophosphorylase serum to the immobilised lacZ fusion protein (Figure III.21B).

(2). Clone WE:AGA.7 cDNA acts as a template for the *in vitro* transcription of a truncated ADP-glucose pyrophosphorylase mRNA. Immunoprecipitation of the *in vitro* translation products of this mRNA occurs with anti-spinach leaf ADP-glucose pyrophosphorylase serum (Figure III.24). Immunoprecipitation of these translation products is successfully competed for by purified wheat endosperm ADP-glucose pyrophosphorylase, indicating that the *in vitro* translation products of WE:AGA.7 mRNA are ADP-glucose pyrophosphorylase polypeptides.

(3). Amino acid residues 1-26 encoded by Clone WL:AGA.1 have 70% identitiy to residues 37-63 of the amino acid sequence derived from a rice endosperm ADP-glucose pyrophosphorylase cDNA clone (Preiss *et al*, 1987; Figure III.33). Amino acid residues 151-170 encoded by clone WE:AGA.7 have 65% identity with residues 1-19 of the amino acid sequence derived from a rice endosperm ADP-glucose pyrophosphorylase cDNA clone (Preiss *et al*, 1987; Figure III.33). In addition, amino acid residues 62-119 encoded by clone WE:AGA.7 have 50% identity with residues 11-68 of *E.coli* ADP-glucose pyrophosphorylase, which has been designated as the binding site for fructose-1,6-bisphosphate (Parsons and Preiss, 1978b; Figure III.33).

The cDNA inserts of wheat ADP-glucose pyrophosphorylase clones WL:AGA.1 and WE:AGA.7 hybridise to a 1.8 kb wheat endosperm mRNA species and a 1.7 kb wheat leaf mRNA species. These size estimates are in close agreement with those obtained by Krishnan *et al* (1986). The estimated size of the wheat endosperm ADP-glucose pyrophosphorylase mRNA is sufficient to contain the structural information required to code for a 61 kD protein, the size of the largest primary translation product of ADP-glucose pyrophosphorylase mRNA (see Section III.4.B).

Unfortunately, none of the cDNA clones isolated is full-length. Based upon the current size estimate for wheat leaf ADP-glucose pyrophosphorylase mRNA, the 950 bp WL:AGA.1 cDNA contains only 55% of the complete sequence. The largest wheat endosperm ADP-glucose pyrophosphorylase cDNA WE:AGA.7 is 1798 bp in length and comigrates in DMSO/glyoxal gels with wheat endosperm ADP-glucose pyrophosphorylase mRNA. However, coupled *in vitro* transcription and translation of this cDNA produces truncated ADP-glucose pyrophosphorylase polypeptides, suggesting that the sequence lacks a functional translation start site. The sizes of these truncated polypeptides can be explained in terms of translational initiation at internal ATG codons in the WE:AGA.7 mRNA. Kozak (1986) suggested that sequences flanking the first ATG codon in eukaryotic mRNAs decide whether the codon is in an optimum context for recognition by ribosomal 40S subunits. The optimal context sequence derived for eukaryotic mRNAs is CCACCATGG (Kozak, 1986) and for plant mRNAs is TAAACAATGGCT (Joshi, 1987a). The WE:AGA.7 sequence lacks a consensus sequence for "scanning translational initiation sites" of plant mRNA (Joshi, 1987a) or eukaryotic mRNAs in general (Kozak, 1986), in the context of the first ATG codon at position 94. Thus, it is reasonable to assume, that in the absence of a translational start site in optimum context, *in vitro* translation of WE:AGA.7 mRNA would initiate at any ATG codon in the sequence.

The WE:AGA.7 cDNA encodes a polypeptide of 55,500 molecular weight, which exceeds the size of the ADP-glucose pyrophosphorylase primary translation product. Since the cDNA is not full-length then this sequence is probably homologous to the mRNA encoding the 61 kD ADP-glucose pyrophosphorylase primary translation product identified in Section III.4.B.

2. Identification of a putative transit peptide sequence encoded by WE:AGA.7 cDNA

The amino acid sequence of the first 33 residues encoded by clone WE:AGA.7 exhibits properties similar to the transit peptides of chloroplast proteins (Colman and Robinson ,1986) and to the transit peptide of an amyloplast protein, granule-bound starch synthase (Klösgen *et al*, 1986). The sequence is rich in hydroxylated and basic amino acids, especially arginine which occurs every 4-5 residues in the sequence. In the first 33 amino acids of the WE:AGA.7 polypeptide sequence and also the granule-bound starch synthase transit peptide sequence (Klösgen *et al*, 1986), the alanine and arginine contents are approximately 20%. However, the WE:AGA.7 sequence also has a very high proline content of 15%. The same amino acids which are absent, or of low abundance in the N-terminus of the WE:AGA.7 polypeptide, are also under-represented or absent in the transit peptide sequence of the granule-bound starch synthase protein (Klösgen *et al*, 1986). There is a low abundance of methionine, cysteine, histidine, glutamate and leucine in the first 33 amino acid residues of the WE:AGA.7 polypeptide sequence, while aspartate, phenylalanine, isoleucine, lysine, asparagine, valine, tryptophan and tyrosine are absent. Despite the similarity in amino acid content of the putative ADP-glucose pyrophosphorylase transit peptide and the granule-bound starch synthase transit peptide, the amino acid sequences are not related. There is also no identity between the putative ADP-glucose pyrophosphorylase transit peptide and the transit peptide sequences of nuclear-encoded chloroplast proteins, for example the small subunit of Rubisco (Mazur and Chui, 1985) and the 16 kD polypeptide of the oxygen-evolving complex (Jansen *et al*, 1987).

The preponderance of basic amino acids in the putative ADP-glucose pyrophosphorylase transit sequence produces a very hydrophilic N-terminal domain in the WE:AGA.7 encoded polypeptide (Figure III.31B). The secondary structure predictions of Chou and Fasman (1978) for the transit sequence indicate that there is an α-helical structure between Pro_4 and Pro_{15} (Figure III.31B). Between residues Pro_{15} and Pro_{28} are three β-turns, and so this region of the protein may not be held in any strict conformation (Figure III.31B).

The exact processing site for the pre-ADP-glucose pyrophosphorylase polypeptide has not been determined in these studies and its identification will require N-terminal amino acid sequence analysis of the mature protein from wheat endosperm. However, cleavage of the precursor polypeptide between Met_{32} and Cys_{33} would produce a protein of 51.8 kD, the approximate size of the mature ADP-glucose pyrophosphorylase polypeptide. Furthermore, the chloroplast-specific transit peptide of the small subunit of Rubisco is also removed at the boundary between a cysteine and a methionine residue (Mazur and Chui, 1985), while the amyloplast-specific starch synthase transit peptide is removed at the boundary between a cysteine and an alanine residue (Klösgen *et al*, 1986). It would be of interest to determine whether there is sufficient information present in the truncated ADP-glucose pyrophosphorylase transit peptide encoded by clone WE:AGA.7, to direct the import of proteins into isolated wheat endosperm amyloplasts. However, in order to examine this, site-directed mutagenesis of the clone would need to be performed to introduce an ATG codon in optimum context for translation (Kozak, 1986; Joshi, 1987a) at the start of the WE:AGA.7 nucleotide sequence. An alternative to this approach would be to clone the entire cDNA into a vector containing both a promoter for RNA polymerase and an ATG codon in optimum context for translation. In this case, the ATG codon would need to be in the same reading frame as the introduced cDNA sequence.

3. Organisation of wheat ADP-glucose pyrophosphorylase genes

Two major sub-family divisions of wheat ADP-glucose pyrophosphorylase genes have been identified in the current study. The wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase cDNAs cross-hybridise only under low-stringency hybridisation conditions, indicating that the two ADP-glucose pyrophosphorylase sub-families are expressed in an organ-specific manner. The observation that expression of ADP-glucose pyrophosphorylase genes is organ-specific is consistent with previous enzyme kinetic data (Fentem and Catt, I.C.I. Plant Biotechnology Group, Runcorn, cheshire, U.K.; personal communications) and immunological data (see Section III.3.B). Within multigene families, the occurrance of sub-families is quite common. Sub-families have been identified for the maize zein genes (Heidecker and Messing, 1986), wheat glutenin subunit genes (Thompson *et al*, 1983), maize sucrose synthase genes (Gupta *et al*, 1988), wheat α-amylase genes (Lazarus *et al*, 1985), small subunit of Rubisco genes (Dean *et al*, 1985), chlorophyll a/b binding proteins (Dunsmuir, 1985), chalcone synthase genes (Koes *et al*, 1987) and soybean β-tubulin genes (Guiltinan *et al*, 1987). The degree of relatedness between wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase gene sub-families is quite small, since there is no detectable cross-hybridisation between the leaf and endosperm ADP-glucose pyrophosphorylase gene fragments in Southern blot hybridisations, even under low-stringency hybridisation conditions. Moreover, the 55.5% identity between wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase cDNA sequences is less than the identities reported for sequences of other gene sub-families. The small degree of identity between wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase cDNAs is also evident at the protein level, since wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase polypeptides are only 55% identical. In consideration of the overall similarities in secondary structure predictions for the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase polypeptides, a conservation of structural features in the protein is probably more important than conservation of primary protein structure in the maintenance of enzyme function.

Southern blot hybridisation analysis of wheat genomic DNA indicates that the ADP-glucose pyrophosphorylase gene family is quite small, consisting of approximately 2-5 genes in each sub-family. It is possible that some of the restriction fragments which hybridised to the cDNAs in these experiments were derived from pseudogenes, and so the number of genes capable of being expressed in wheat endosperm and wheat leaves *in vivo*, may be less than this upper limit on the total number of genes. Recently Krishnan *et al* (1986) has estimated that there are three rice ADP-glucose pyrophosphorylase genes related to the rice endosperm ADP-glucose pyrophosphorylase cDNA. However, since the organisation of the rice ADP-glucose pyrophosphorylase gene family has not been fully examined, we do not know whether this estimate includes leaf-specific sequences.

The presence of organ-specific isoenzymes of ADP-glucose pyrophosphorylase has also been observed in the leaves and endosperm of maize and rice (Krishnan *et al*,1986), as well as wheat. Presumably, there is some selective advantage to the maintenance of this organ-specificity. In view of the differential sensitivity of wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase enzymes to activation by 3-phosphoglycerate and inhibition by orthophosphate (Fentem and Catt, personal communications), organ-specific expression of ADP-glucose pyrophosphorylase genes may allow the plant to differentially regulate starch biosynthesis in the leaves and endosperm.

Further subdivision of the wheat endosperm ADP-glucose pyrophosphorylase gene sub-family has been carried out at the DNA level using restriction enzyme mapping data. There is polymorphism around the *Sst*I and *Bgl*II sites in the wheat endosperm ADP-glucose pyrophosphorylase cDNAs. On this basis, clones WE:AGA.1, WE:AGA.6, WE:AGA.7 represent one class of endosperm gene while clones WE:AGA.3, WE:AGA.5 represent a second class of endosperm gene. The two wheat endosperm ADP-glucose pyrophosphorylase cDNAs, WE:AGA.3 and WE:AGA.7 have been sequenced and found to be very closely related, with 96% identity between the nucleotide sequences in the open reading frame. Only six of the eighteen nucleotide substitutions within the open reading frame produce amino acid substitutions. Miyata *et al* (1982) have reported that silent base substitutions accumulate in several animal genes at the rate of 5.37×10^{-9} substitutions/year. Within the overlapping regions of the WE:AGA.3 and WE:AGA.7 open reading frames, there are 369 nucleotide positions for which substitution is potentially silent. The 12 silent mutations actually observed between these sequences therefore correspond to an approximate evolutionary distance of 6.05×10^{6} years. This would imply that the corresponding genes have been separated for approximately 3×10^{6} years, assuming equal rates of mutation in the two genes. However, it is also possible that, as a result of plant breeding, the silent mutation rate in wheat genes is greater than in animal genes. As wheat has only been cultivated for approximately 6×10^{3} years, then silent mutations in the endosperm ADP-glucose pyrophosphorylase genes would have accumulated at a rate of 2.7×10^{-6} substitutions/year on average, if the divergence has occurred as a result of intensive plant breeding. Regardless of the time when the wheat endosperm ADP-glucose pyrophosphorylase genes diverged, the 72% identity between the 3'-untranslated regions of WE:AGA.3 and WE:AGA.7 cDNAs indicates that the genes almost certainly arose as a result of a gene duplication.

4. Functional protein domains of ADP-glucose pyrophosphorylases

Regions of the *E.coli* and spinach leaf ADP-glucose pyrophosphorylase polypeptide sequences have been identifed previously as substrate, activator or inhibitor binding sites (Parsons and Preiss, 1978a, 1978b; Larsen *et al*, 1986; Lee and Preiss, 1986; J. Preiss, personal communications). The derived amino acid sequences of wheat leaf and wheat endosperm ADP-glucose pyrophosphorylases have been compared with substrate, activator and inhibitor binding sites identified on other ADP-glucose pyrophosphorylase enzymes. These binding sites form five protein domains, indicated in the protein alignment shown in Figure III.33.

1. The fructose-1,6-bisphosphate binding site:

The allosteric activator (fructose-1,6-bisphosphate) binding site of *E.coli* ADP-glucose pyrophosphorylase, at the N-terminus of the protein close to the Lys_{90} residue, is indicated in Figure III.33 as peptide segment I (Parsons and Preiss, 1978b; see also Section I.4.B). The amino acid sequence in wheat endosperm ADP-glucose pyrophosphorylase related to the fructose-1,6-bisphosphate binding site of the *E.coli* enzyme (Parsons and Preiss, 1978b) has been identified. The region from residue 78-100 has 14 out of the 23 amino acids conserved between wheat endosperm and *E.coli* sequences. However, there are major alterations at several amino acid positions, involving the substitution of charged residues in the *E.coli* sequence for hydrophobic or amphipathic residues in the wheat endosperm sequence , for example Arg_{80}-Thr_{80}, Lys_{85}-Phe_{85}, Asp_{86}-Pro_{86}, Lys_{90}-Thr_{90}, Lys_{93}-Thr_{93}, His_{97}-Pro_{97} (Figure III.33). Since pyidoxal-5'-phosphate binds at

Lys_{80} in *E.coli* ADP-glucose pyrophosphorylase, and fructose-1,6-bisphosphate binds in this region also (Parsons and Preiss, 1978a, 1978b), this binding site is probably not functional in the wheat endosperm protein.

The secondary structure prediction of Chou and Fasman (1978) for this region of the wheat endosperm sequence indicates β-turns between residues 78-81 and 94-100, with an α-helix between residues 82-93 (Figure III.31B). This prediction differs significantly from the secondary structure prediction of the allosteric activator site of *E.coli* ADP-glucose pyrophosphorylase. In the *E.coli* protein sequence, the preponderance of proline residues in close proximity to Lys_{80} is believed to prevent the formation of any secondary structure in this allosteric site (Parsons and Preiss, 1978b).

Parsons and Preiss (1978b) suggested that arginine and lysine residues were probably involved in binding the allosteric activators fructose-1,6-bisphosphate and pyridoxal-5'phosphate to the *E.coli* enzyme, via an ionic interaction with the phosphate moieties of these metabolites. Moreover, Preiss and Walsh (1981) suggest that an important aspect of the mechanism of allosteric activation of the *E.coli* enzyme might be the neutralisation of basic groups on the protein. This would be achieved by either phosphate, carboxyl, or aldehydic groups on the activator molecule capable of forming a Schiffs base with the amino groups of lysine and arginine residues. Thus, in the wheat endosperm sequence related to the allosteric site of *E.coli* ADP-glucose pyrophosphorylase, there is no opportunity for Schiff base formation between fructose-1,6-bisphosphate and the protein domain. This finding is consistent with the lack of detectable activation of wheat endosperm ADP-glucose pyrophosphorylase by fructose-1,6-bisphosphate (Fentem and Catt, personal communications). It would be instructive to analyse this protein sequence in the wheat leaf ADP-glucose pyrophosphorylase enzyme, since fructose-1,6-bisphosphate is able to activate plant leaf ADP-glucose pyrophosphorylases (Preiss and Levi, 1980). Unfortunately, the wheat leaf ADP-glucose pyrophosphorylase cDNA sequence does not extend far enough into the 5'-coding region to contain sequences of the fructose-1,6-bisphosphate binding site. We cannot be certain, therefore, whether allosteric activation of leaf ADP-glucose pyrophosphorylases by fructose-1,6-bisphosphate is achieved by binding of the activators to the same site as in the *E.coli* enzyme.

2. Inhibitor binding sites: The three 5'-AMP binding domains on *E.coli* ADP-glucose pyrophosphorylase (Larsen *et al*, 1986) are well-conserved in the plant enzymes also. As noted in Section I.4, there is functional overlap between two of the inhibitor binding sites and both the substrate binding site and fructose-1,6-bisphosphate binding site of the *E.coli* enzyme. The third inhibitor binding site in the *E.coli* ADP-glucose pyrophosphorylase enzyme, peptide segment IV in Figure III.33, does not overlap with any other binding sites (Larsen *et al*, 1986). Within this peptide segment, the sequence DFGXXXXP is conserved between all sequences examined (Figure III.33). There is a greater similarity between the plant sequences, for which the consensus DFGSEXXPXA exists between residues 315-324 (Figure III.33). The difference between *E.coli* and plant ADP-glucose pyrophosphorylase proteins with respect to this sequence may reflect the low affinity of plant

enzymes for 5'-AMP compared to the *E.coli* enzyme. Since plant enzymes are inhibited by orthophosphate rather than by 5'-AMP, and the ADP-glucose pyrophosphorylase enzymes of Enterobacteria are inhibited by 5'-AMP and only weakly inhibited by orthophosphate (Preiss and Walsh, 1981), we might expect these enzymes to contain different inhibitor sites. Consequently, we cannot be certain if the identity between plant and *E.coli* sequences in the regions of the 5'-AMP binding sites is of any functional significance.

3. Substrate binding sites: Two substrate binding sites of the *E.coli* ADP-glucose pyrophosphorylase (Parsons and Preiss, 1978b; Lee and Preiss, 1986), designated as peptide segment II and peptide segment III in Figure III.33, are well-conserved in the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase sequences. The region between residues 166-173 (peptide segment II, Figure III.33) contains a consensus sequence of WXXGTADA, obtained from a comparison of wheat endosperm, rice endosperm and *E.coli* protein sequences in this region. In all cases amino acid position 167 is occupied by an aromatic hydrophobic residue, either tyrosine or phenylalanine.

Binding of the adenine ring of ATP and ADP-glucose to Tyr_{167} of *E.coli* ADP-glucose pyrophosphorylase (peptide segment II, Figure III.33) has been implicated by the resistance of Arg_{168} to protease digestion following labelling of the enzyme with $[^{14}C]$8-azido ADP-glucose or $[\alpha-^{32}P]$8-azido ATP (Lee and Preiss, 1986). Tyrosine has been found in association with an adenine ring in several other enzymes, including pig muscle adenylate kinase, bovine mitochondrial F_1-ATPase and porcine heart cAMP-dependent protein kinase (cited in Larsen *et al*, 1986). It is possible that hydrophobic stacking interactions are involved in the co-ordination of the adenine ring with the enzyme. Since phenylalanine, present in the rice and wheat endosperm enzymes, also contains an aromatic side chain there is no reason to believe that this residue would not also be able to co-ordinate the adenine ring of ATP and ADP-glucose. Thus, the maintenance of an aromatic, hydrophobic residue at position 167 in ADP-glucose pyrophosphorylase may be very important for enzyme function.

Comparison of ADP-glucose pyrophosphorylases within the region aligned to peptide segment III o. the *E.coli* enzyme (Figure III.33), reveals a consensus sequence of FXEXP for residues 252-256. Peptide segment III of the *E.coli* enzyme (Figure III.33) binds the photoaffinity substrate analogue, [^{14}C]8-azido-ADP-glucose (Lee and Preiss, 1986). In addition, binding of pyridoxal-5'- phosphate to Lys_{255} of the *E.coli* ADP-glucose pyrophosphorylase is prevented by ADP-glucose (Parsons and Preiss, 1978b), indicating that the substrate binding site is nearby. Although pyridoxal-5'-phosphate forms a Schiffs base with the ϵ-amino group of lysine, it is unlikely that Lys_{255} is itself involved in substrate binding since it is not strictly conserved in the plant ADP-glucose pyrophosphorylase sequences: Clone WE:AGA.7, for example encodes a glutamine residue at position 255. However, Phe_{251} is strictly conserved in all of the ADP-glucose pyrophosphorylase sequences examined, indicating that this residue might be involved in binding ATP and ADP-glucose, also by hydrophobic interaction between the planar aromatic ring of the phenylalanine side-chain and the adenine ring of the substrate molecules. Nevertheless, an alternative mechanism involving ionic interaction between the phosphate moiety of ATP or ADP-glucose and the amino group of

Arg_{287} (or Lys_{287}) cannot be ruled out..

In the putative substrate binding domains of ADP-glucose pyrophosphorylase enzymes there is no amino acid identity with the consensus sequence of Higgins *et al* (1986) for ATP binding sites. That consensus sequence (GXXXXGKS) has been shown by crystallographic analysis to form the phosphate binding region (Pai *et al*, 1979, cited in Higgins *et al*, 1986). A variant of this sequence, GDOLAEGKV in the wheat leaf sequence and SRLMSEGKV in the wheat endosperm sequences is located at position 460-468 in Figure III.33.

4. The 3-phosphoglycerate binding site: Peptide segment V in Figure III.33 has been identified as a 3-phosphoglycerate binding site in the spinach leaf ADP-glucose pyrophosphorylase enzyme (J. Preiss, personal communications; see also Section I.4.B). The sequences shown in Figure III.33 were compared to amino acid sequence of the putative 3-phosphoglycerate binding site on spinach leaf ADP-glucose pyrophosphorylase (see Figure I.5).

Different activation kinetics of the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase enzymes are not explained in terms of altered binding sites for 3-phosphoglycerate, since there is no obvious correlation between enzyme activation and conservation of the 3-phosphoglycerate binding site. For example, the wheat endosperm enzyme is not activated by 3-phosphoglycerate (Fentem and Catt, personal communications), while the rice endosperm enzyme is only activated 8-fold by 20 mM 3-phosphoglycerate ($A_{0.5}$= 5 mM; Lee and Wang, 1985). In contrast, the spinach leaf enzyme is stimulated 20-fold by less than 150 μM 3-phosphoglycerate concentration ($A_{0.5}$= 51 μM; Copeland and Preiss, 1981) and the wheat leaf enzyme is activated 14.5-fold by 3-phosphoglycerate, with an $A_{0.5}$ value of 100 μM (MacDonald and Strobel, 1970). Despite these observations, the wheat leaf and spinach leaf ADP-glucose pyrophosphorylase proteins differ in 8 out of the 13 residues contained within the putative 3-phosphoglycerate binding site. The wheat endosperm sequences also have limited identity to the spinach sequence, differing in 7 out of 13 residues. There is a greater identity between the spinach leaf and rice endosperm sequences in this region, with strict conservation of 11 out of 13 amino acids. In addition, although the primary sequences of wheat endosperm and wheat leaf ADP-glucose pyrophosphorylases differ in 6 of the 13 residues of the 3-phosphoglycerate binding site, the Chou and Fasman (1978) secondary sructure predictions for this sequence are nearly identical (Figure III.34).

Since 3-phosphoglycerate is able to prevent the inhibition of the wheat endosperm ADP-glucose pyrophosphorylase by orthophosphate (Fentem and Catt, personal communications), this suggests that the enzyme possesses a functional 3-phosphoglycerate binding site. A consensus sequence for the binding of 3-phosphoglycerate, between residues 515-522 is SGIXXXXK, obtained by a comparison of all available plant ADP-glucose pyrophosphorylase sequences. Residue Lys_{522} is strictly conserved in all sequences, and is involved with binding of pyridoxal-5'-phosphate, and probably also 3-phosphoglycerate, to the spinach leaf ADP-glucose pyrophosphorylase enzyme (J. Preiss, personal communications). Amino acid positions 518 and 519 are occupied by valine or threonine in all of the plant sequences (Figure III.33), while amino acid position 520 is occupied by either valine or isoleucine (Figure III.33). In addition, aspartate or asparagine occupies position 523, while alanine or serine occupies position 524 of the putative 3-phosphoglycerate binding site (Figure III.33).

Thus, it is likely that alterations between wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase amino acid sequences in regions other than the putative 3-phosphoglycerate binding site, are responsible for the observed differences in allosteric properties of these enzymes. Since the amino acid sequences of these two enzymes are only 55% identical, any of the observed amino acid alterations might explain their different allosteric properties. This would suggest that the wheat endosperm enzyme might not be able to undergo the necessary conformational changes required to convert it to a more active form in the presence of 3-phosphoglycerate.

The consensus sequence for 3-phosphoglycerate binding is not identical to any region of the *E.coli* ADP-glucose pyrophosphorylase sequence (Figure III.33). The absence of a site for optimal binding of 3-phosphoglycerate is consistent with the observation that the level of activation of the *E.coli* enzyme obtained with 3-phosphoglycerate is 10% or less of the maximum stimulation achieved by fructose-1,6-bisphosphate (Preiss and Walsh, 1981). Thus, it is likely that the marginal activation of this enzyme by 3-phosphoglycerate is achieved by weak binding of the metabolite to an alternative site, such as the fructose-1,6-bisphosphate binding site.

SECTION IV- CONCLUSIONS

ORGAN-SPECIFIC POLYMORPHIC EXPRESSION OF WHEAT ADP-GLUCOSE PYROPHOSPHORYLASE GENES

The demonstration by Fentem and Catt (personal communications) that the wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase enzymes exhibit different allosteric properties with respect to activation by 3-phosphoglycerate and inhibition by orthophosphate, has been extended in the current study to a characterisation of the organ-specific isoenzymes at the protein and DNA levels. These ADP-glucose pyrophosphorylase isoenzymes have been found to contain different molecular weight subunits. In addition, cDNA clones encoding wheat leaf and wheat endosperm ADP-glucose pyrophosphorylases have been isolated and characterised. Studies on the organisation of wheat ADP-glucose pyrophosphorylase genes indicates that they are organised into a small gene family, consisting of at least two organ-specific gene sub-families, expressed in the leaves and developing endosperm respectively. Nucleotide sequence analysis of wheat leaf and wheat endosperm ADP-glucose pyrophosphorylase cDNAs indicates that there is only 55% identity between the open reading frames of these organ-specific genes. The occurrence of organ-specific ADP-glucose pyrophosphorylase genes which are conserved to such a small extent is consistent with the existence of different biochemical pathways of starch synthesis in leaves and endosperm of wheat (see Section I.2.A and Section I.3.A.) and different modes of regulation of starch biosynthesis in these organs (see Section I.2.B and Section I.3.B).

The analysis carried out on functional protein domains of ADP-glucose pyrophosphorylases has increased our knowledge of the amino acid sequence motifs required for binding of various metabolites. It now seems apparent that the lack of *in vitro* allosteric activation of wheat endosperm ADP-glucose pyrophosphorylase is probably due to the inability of this enzyme to undergo conformational transitions necessary to convert it to a more active form. This is suggested by kinetic studies of Fentem and Catt (personal communications) and nucleotide sequence data which indicate that the 3-phosphoglycerate binding site of wheat endosperm ADP–glucose pyrophosphorylase is functional.

A future direction for the analysis of organ-specific ADP-glucose pyrophosphorylase gene expression must certainly be the isolation and characterisation of the promoter sequences responsible for this organ-specificity, presumably located at the 5'-termini of the leaf and endosperm ADP-glucose pyrophosphorylase genes. The isolation and characterisation of cDNAs presented in the current study will greatly facilitate such an analysis. The 5'-untranscribed regions of eukaryotic genes contain the sequence motifs responsible for organ-specific gene expression and developmental or environmental regulation of gene expression, in addition to the TATA box and CAAT box sequences which are essential elements of all promoters. Enhancer sequences are also present in the 5'-untranscribed regions of genes, which are capable of conferring strong transcriptional activation on the gene (Serfling *et al*, 1985). Although stable transformation and regeneration of cereals is not yet a reality, a definition of the sequences responsible for the organ-specific expression of the ADP-glucose pyrophosphorylase gene family may be carried out using deleted promoter constructs linked to a bacterial-CAT reporter gene in stably-transformed tobacco plants. There are not many reports in the literature on the recognition of critical sequences for organ-specific expression of wheat genes in tobacco and so there is always the risk that correct expression of monocot genes will not occur in tobacco plants. However, Nagy *et al* (1986) have observed that the wheat chlorophyll a/b-binding protein gene is expressed only in the leaves of transformed tobacco, suggesting that the factors conferring organ-specific gene expression may be conserved between monocots and dicots. Alternatively, it may be possible to carry out an analysis of deleted promoter constructs using a transient expression system based on wheat endosperm and wheat leaf protoplasts. This sort of functional analysis has recently been conducted, using a maize protoplast transient expression system to elucidate the regulatory sequences responsible for anaerobic induction of the alcohol dehydrogenase genes and the aldolase genes of maize (Dennis *et al*, 1987; Walker *et al*, 1987; Peacock *et al*, 1987).

The identification and characterisation of cDNAs encoding wheat endosperm ADP-glucose pyrophosphorylase provides us with the opportunity to study the contribution of this enzyme to the regulation of starch biosynthesis in wheat endosperm, and perhaps to subsequently modify the levels of starch accumulated during grain filling. If the stable transformation and regeneration of cereals does become a reality in the near future, the introduction of additional copies of wheat endosperm ADP-glucose pyrophosphorylase genes should produce greater levels of the enzyme in wheat endosperm amyloplasts, assuming that the higher level of gene expression is not offset by increased enzyme turnover. Increased gene expression may also be elicited using the molecular approach, by introducing multiple copies of enhancer sequences into the 5'-untranscribed region of one gene. Expression of the anaerobically regulated alcohol dehydrogenase and aldolase genes, for example, is positively correlated to the number of anaerobic regulatory elements (enhancers) found in the promoter region (Dennis *et al*, 1987). Thus, it may be possible to increase expression of wheat endosperm ADP-glucose pyrophosphorylase genes by producing transformed plants with altered copy number of enhancer sequences in the ADP-glucose pyrophosphorylase gene promoter. If this enzyme has a high control strength in wheat endosperm starch biosynthesis and therefore contributes to the net regulation of carbon flux to starch, in wild-type plants, then the rate of starch biosynthesis would be expected to increase in the transformed plants.

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